Chemical ecology of *Chaoborus* as predator and prey: Effects of infochemicals and food quality on inducible defences and gene expression in *Daphnia*

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"There is always another secret"

- Brandon Sanderson

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General introduction and aim of the study

Daphnia from the order Cladocera are an important keystone species and model organisms in freshwater ecology. They occur in all types of standing water bodies, ranging from large lakes to very small and even temporary ponds (Ebert 2005) as well as in all climatic zones from the arctic (Edmondson 1955) over tropics to the desert (Dodson 1985). *Daphnia* have a very short generation time and reproduce mostly parthenogentic, which allows for the cultivation of clonal lineages of animals sampled under specific environmental conditions.

Beyond *Daphnia*`s good applicability for research, their ecological position within freshwater food webs is even more important. *Daphnia* are suspension feeders, which filter particles unselectively within the size range from 1 µm to approximately 30 µm from the surrounding water. Their diet consists mainly of unicellular phytoplankton, but also smaller colonies can be consumed. Since they feed unselectively, *Daphnia* are highly dependent on the composition of the given phytoplankton community in their habitat (Müller-Navarra & Lampert 1996) and are not able to exclude low quality food or even harmfully toxic algae and cyanobacteria from their diet.

As primary consumers they are in a key position crucial for the energy transfer from primary producers to higher trophic levels. *Daphnia* serve as a food source for a highly diverse group of planktivorous predators ranging from invertebrate aquatic insects like *Notonecta* (McArdle & Lawton 2008) or aquatic insect larvae like *Chaoborus* (Swift & Fedorenko 1975) over vertebrates like tadpoles (Hamilton *et al.* 2012) to fish (Luecke *et al.* 1990).

Further, *Daphnia* show a high degree of phenotypic plasticity, which means that individuals from the same clonal line (of the same genotype) can exhibit strongly differentiating phenotypes concerning their behavior, life-history or morphology dependant on the environmental conditions. It was shown, for example, that *Daphnia* suffering hypoxia can

raise the concentration of haemoglobin in their hemolymph to develop a higher tolerance towards environmental hypoxia, causing animals to change their colour from pale transparent to red (Kobayashi & Hoshi 1982). Furthermore, Milla & Korhola (2002) showed, that arctic *Daphnia* can adjust their pigment synthesis according to the underwater ultraviolet irradiance, exhibiting low levels of melanin synthesis during the arctic winter and peak levels after the ice breakup in spring. In addition, Ghadouani & Pinel-Alloul (2002) demonstrated that daphnids responded to increased concentrations of inedible phytoplankton by increasing the mesh size of their filter apparatus.

A widely studied phenotypically plastic trait in *Daphnia* is the induction of defences against predators. An inducible defence, opposed to a constitutional defence, is only expressed when needed, in order to minimize putative evolutionary costs like a reduced growth, reproductive output and survivorship (Harvell 1990). This should be adaptive, if the induction of the defence is indeed associated with costs and there is a temporally varying threat of predation. The inducibility of a defence allows a prey organism to derive a benefit from the defence when needed but avoiding the costs if no predator is present. However, an inducible defence needs a reliable and predator specific cue which indicates the presence of a predator and provides a fitness advantage for the receiving prey species and a fitness disadvantage for the emitting predator. These predator cues are called kairomones, which are chemical substances that have been shown to be released by their respective predator. Although the chemical identity of no kairomone in freshwater food webs could be uncovered yet, some of these kairomones have been preliminarily characterized. Tollrian & Von Elert (1994) demonstrated the kairomone released by *Chaoborus* to be of low molecular weight and extractable with lipophilic sorbents, which allowed an enrichment of the bioactive substance(s) via C_{18} solid phase extraction.

Daphnia is known to exhibit a wide range of different inducible defences due to the presence of kairomones from a vast number of different predators. Loose (1993) showed that, during

daytime light conditions, a gradual increase in fish abundance induced a chemically mediated increase in the average residence depth of *Daphnia*, resulting in a habitation at darker light conditions. This benefits *Daphnia* in reducing mortality due to visually hunting fish. In contrast to this, kairomone released by the invertebrate *Chaoborus*, the aquatic larvae of the phantom midge, has been shown to induce an upward migration in *Daphnia* during daytime. This serves equally to reduce spatial overlap and thus encounter probability with this predator, since *Chaoborus* has been shown to remain at deeper water layers during daytime (Ramcharan *et al.* 1992).

Daphnia has been shown to undergo a resource allocation and alter its life-history when exposed to fish kairomones, which results in a reduced size at first reproduction (Weider & Pijanowska 1993). This is advantageous for *Daphnia* since smaller body sizes increase the chance of survival, because visually hunting predators like fish are size selective for larger prey (Brooks & Dodson 1965). On the other hand, Riessen (2012) showed that *Chaoborus* kairomone exposure during embryonic development led to an increase in body size in *Daphnia pulex* neonates. As *Chaoborus* is a gape limited sit-and-wait predator, strike efficiencies are negatively correlated with prey body size (Riessen & Trevett-Smith 2009).

Additionally, *Chaoborus* kairomone is known to induce various morphological defences in different *Daphnia* species. These alterations of the carapace range from elongated tail spines in *D. lumholtzi* (Agrawal 2001) over enlarged heads, so called helmets, in *D. galeata* (Oda *et al.* 2007) to enlarged crests in *D. longicephala* (Laforsch *et al.* 2006). In *D. pulex, Chaoborus* kairomone has been shown to induce so called “neck-teeth”, which are spike like structures at the back of head (Krueger & Dodson 1981). Additionally, the morphology of the head that carries these neck-teeth can change as a whole, resulting in the formation of an angular shape (“neck-keel”) or even a protuberance called “pedestal” (Tollrian 1993).

These morphological defences have been shown to strongly reduce mortality due to *Chaoborus* predation by increasing the escape efficiency of captured prey (Havel & Dodson

1984). As *Chaoborus* is a gape-limited predator, only individuals below a certain body size are susceptible to predation. As a consequence, only those instars which are susceptible exhibit the induction of morphological defences (Riessen & Trevett-Smith 2009). However, individuals have also been reported to be too small to derive a benefit from the induction of neck-teeth, creating a size window wherein neck-teeth provide an energetically worthwhile benefit for *Daphnia* (Riessen & Trevett-Smith 2009). Furthermore, the strength of neck-teeth induction depends on the abundance of the predator in the surrounding habitat, leading to a Michaelis-Menten-like dose-response curve dependant on kairomone concentration (Tollrian 1993).

A lot of studies have focused on quantifying the costs of neck-teeth (e. g. Riessen & Sprules (1990), Walls *et al.* (1991), Hammill *et al.* (2008) and Riessen (2012) or the fitness benefit obtained by neck-teeth induction ((Havel & Dodson 1984), (Riessen & Trevett-Smith 2009)). However, none of these studies addressed the fact that *Daphnia* is an unselective suspension feeder underlying the constantly changing composition of the naturally heterogeneous phytoplankton community (Sommer *et al.* 1986). Instead, most studies assume constantly beneficial food conditions by cultivating *Daphnia* cultures at unchanging food conditions resulting in an algal diet of constant nutritional quality (*Chlamydomonas* sp., *Scenedesmus* sp., *Chlorella vulgaris*, *Pseudokirneriella* sp.,). In nature, however, the phytoplankton community is made up of a lot of different algal species, but only a limited number of those are nutritionally adequate for zooplankton (Stemberger 1981). But since Daphnids feed unselectively, a mixture of nutritionally adequate as well as inadequate algae is ingested. **Chapter 1** addresses the question if and how differences in algal food quality have an impact on the strength of the induction of morphological defence in *D. pulex*. Therefore, two different *D. pulex* clones, which previously showed a different strength in neck-teeth induction at equal amounts of *Chaoborus* kairomone, were kept on three different food algae of different nutritional quality. I conducted life history experiments coupled with

morphological analyses in order to quantify neck-teeth induction in correlation with maternal food quality. Beyond that, and in the context of the findings of von Elert & Stampfl (2000), who showed that algal food quality was dependent on the specific poly-unsaturated fatty acids (PUFAs), I produced liposomes containing various PUFAs and conducted food supplementation experiments, wherein low quality algae were fed in combination with PUFA-containing liposomes.

This experiment revealed for the first time the influence of maternal food quality on the strength of neck-teeth induction coupled with the effect on life-history, illustrating the importance of the food quality aspect when conducting, interpreting and especially comparing research results of this topic. In addition, I presented a single PUFA, which was shown to be responsible for the upgrade of low quality to high quality food.

In 2011, Colbourne *et al.* (2011) published the completely sequenced genome of *D. pulex*. Furthermore, Nakanishi *et al.* (2014) introduced a method for gene knockout in *Daphnia*. Combined, these two publications offer a means for researchers to understand the induction of neck-teeth on a whole new level. However, suitable candidate genes are required in order to create neck-teeth related knockout mutants in the first place. Miyakawa *et al.* (2010) employed a candidate gene approach and identified a differently expressed set of genes comprised of several morphogenetic factors and genes belonging to the either the juvenile hormone or insulin signalling pathway. However, inaccuracy in their experimental design might indicate false positive correlations between kairomone exposure and gene regulation in the experimental *D. pulex*. In **Chapter 2**, I introduced a novel method by analyzing gene expression of genes derived from Miyakawa *et al.* (2010) in three different *D. pulex* clones which have been shown to differ significantly concerning their strength of neck-teeth induction. Further, I investigated the influence of *Chaoborus* kairomone on the gene expression of two chitin deacetylases, which were reported to be upregulated on proteome level in *D. magna* under the influence of kairomone released by *Triops cancriformis*. Thus,

Chapter 2 provides important and novel insights into already existing gene expression patterns on one hand, while on the other hand providing additional suitable candidate genes for further research.

However, predator-prey relations are not an ecological one-way street. As Dawkins & Krebs (1979) stated, an adaptation in one species (e. g. the prey) may change the selection pressure on another species (e. g. the predator), giving rise to a counter-adaptation. If this occurs reciprocally, an "arms-race" may result. Kotov & Taylor (2011) found daphnid fossils co-occurring with fossils of predaceous phantom midges dating back approximately 145 million years, which suggested a long period of ongoing predator-prey interactions and thus coevolution of both groups. Consequently, and in consideration of Dawkins & Krebs (1979) proposed arms-race, it seems likely that not only *Daphnia* have evolved strategies to reduce mortality due to predation, but that also *Chaoborus* has established ways of increasing the predatory success. **Chapter 3** is concerned with the investigation of the vertical position of *Chaoborus* larvae in the water column depending on the presence or absence of water in which feeding *D. magna* had been incubated. Therefore, I conducted behavioural observations utilizing a plankton organ, which simulated a natural lake with a temperature and light gradient. Additionally, as *Chaoborus* larvae are known to migrate deeper in the water column due to fish kairomones in order to reduce detectability for visually hunting fish (Dawidowicz *et al.* 1990), assays applying both the foraging kairomone of *Daphnia* and the predator kairomone of fish, were performed, to obtain results which are of high ecological relevance.

Chapter 3 shows for the first time that *Daphnia* release a foraging kairomone utilized by *Chaoborus* and that *Chaoborus* larvae are able to differentiate between different kairomones indicated by distinct behavioural responses under different predator and light regimes.

Chapter 1

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Dietary quality affects the strength of a morphological anti-predator defence in *Daphnia*

Abstract

The freshwater grazer *Daphnia* simultaneously has to face seasonally varying levels of predation threat and food quality in nature. Larvae of the phantom midge *Chaoborus* are gape-limited predators, and vulnerability of *Daphnia* against *Chaoborus* predation has been shown to decrease with increasing prey size. Here we hypothesize that low food quality increases the time window that a prey remains vulnerable and that this increases the strength of inducible morphological defence in *D. pulex* in response to predator cues from *Chaoborus*. By varying food quality, we show that a longer time needed to outgrow the vulnerable prey size is related to an increased expression of a morphological anti-predator defence (induction of neck-teeth). By supplementation, we demonstrate that the absence of a single essential polyunsaturated fatty acid in low quality food causes this increase in neck-teeth induction. These data indicate that the strength of a morphological defence in nature does not only depend on the concentration of predator cues, but is strongly modulated by seasonal variations in lipid-mediated food quality.

Introduction

In standing freshwater ecosystems the keystone species *Daphnia* has become a model system for predator-prey interactions. Daphnids feed unselectively on small particles that are suspended in the water, and thus are highly dependent on the composition of the ambient phytoplankton community. (Müller-Navarra & Lampert 1996) have shown that both quantity and quality of *Daphnia* food, provided as natural lake seston, varied seasonally. (Ahlgren *et al.* 1990) demonstrated further, that diets of different algal species caused different growth rates in *Daphnia*, with the two green algae *Scenedesmus acutus* and *Chlamydomonas* sp. supporting rather low somatic growth rates compared to the flagellate *Cryptomonas* sp. By supplementing *Scenedesmus* with different PUFAs (von Elert 2002) showed that the low food quality of this green alga was due to the absence of polyunsaturated fatty acids (PUFAs) from this alga. Within a single lake PUFA-mediated food quality of seston for *Daphnia* has repeatedly been shown to vary considerably throughout the season ((Wacker & von Elert 2001), (Müller-Navarra *et al.* 2000)).

However, differences in food quality are not the only constraint *Daphnia* have to cope with in natural systems. Predation by both vertebrate and invertebrate predators poses a major threat. Larvae of the phantom midge *Chaoborus* are a common predator with highly varying abundances throughout the year (Jeong & Park 2010). Several inducible defences have evolved in *Daphnia* to counteract this predation risk. For example, neonates of *Daphnia pulex* are born at a larger size, when exposed to *Chaoborus* kairomone during embryonic development (Riessen 2012). This seems advantageous, since *Chaoborus* is a gape limited predator with a lower strike efficiency at lager prey sizes (Riessen & Trevett-Smith 2009). Another example for an inducible defence is diel vertical migration. (Dodson 1988) demonstrated, that different *Daphnia* species respond to the presence of larvae of *Chaoborus americanus* by positioning themselves higher in the water column compared to control animals. This behavioural response was supposed to be adaptive, since *C. americanus* larvae

have been shown to remain at lower depth during daytime (Ramcharan *et al.* 1992). Finally, the probably best studied defence is the alteration of *Daphnia* morphology. Different *Daphnia* species are known to develop elongated heads (Laforsch & Tollrian 2004), larger crests (Laforsch *et al.* 2006) or longer tail spines (Kolar & Wahl 1998) as a response to predator kairomones. *D. pulex* has been shown to develop small, spine like structures at the back of the head, so called neck-teeth (Krueger & Dodson 1981) in response to *Chaoborus* kairomone. These neck-teeth have been reported to reduce mortality due to *Chaoborus* predation by up to 50% (Havel & Dodson 1984). In accordance with the assumption that the formation of neck-teeth is associated with costs, for a single *Daphnia* genotype the degree of neck-teeth induction has been shown to increase with kairomone concentration (Fig. 1); however there is pronounced among-genotype variability with respect to the degree of morphological defence at a given kairomone concentration.

Despite the huge number of studies on inducible morphological defences in *Daphnia*, the effect of differences in food quality on the induction of morphological defences has not been investigated yet. Larvae of *Chaoborus* are gape-limited predators and vulnerability of *Daphnia* against *Chaoborus* predation has been shown to decrease with increasing prey size until daphnids have outgrown the size at which they can be ingested (Swift & Fedorenko 1975). Given that differences in food quality affect developmental trajectories and thus developmental rates of juvenile *Daphnia*, it is reasonable to assume that low food quality should result in a longer period of time of being in the vulnerable prey size, which should result in an overall higher predation risk by larvae of *Chaoborus*.

Here we investigate the effect of food quality on the induction of a morphological defence in *Daphnia* in response to *Chaoborus* kairomone. Three different algal diets known to differ in their fatty acid composition were fed to two clones of *D. pulex* that were known to differ in strength of neck-teeth induction in the presence of identical *Chaoborus* kairomone concentrations. We recorded the number of offspring, the time to reach larval instar three and

the strength of neck-teeth induction. We tested for the cause of food quality effects on neck-teeth induction by supplementation of a low quality alga with selected fatty acids using specific liposomes. Finally we investigated whether food quality effects on the juvenile morphological defence in *D. pulex* were due to maternal or juvenile food conditions.

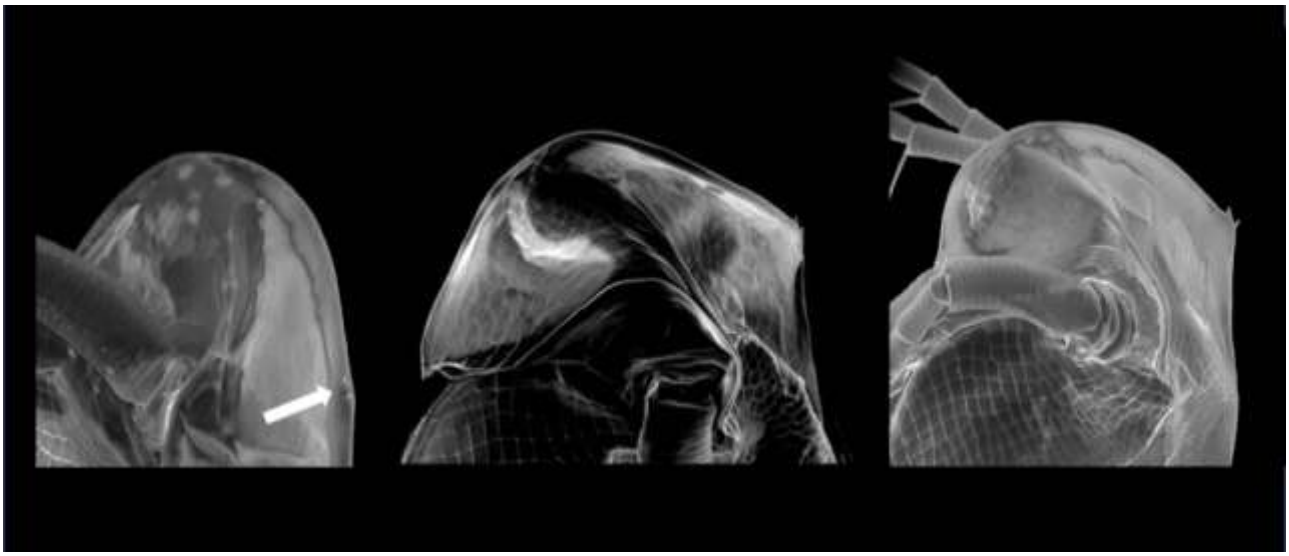

Figure 1: Second instar juveniles of *D. pulex* with increasing neck-teeth induction from left to right. Left: juvenile with two neck-teeth at the back of the head (white arrow) without further defensive structures. Middle: juvenile with a keel and two neck-teeth. Right: juvenile with a pedestral and two neck-teeth. The different degrees of defense represent scores of 20 % (left), 50 % (middle) and 70 % (right).

Material and Methods

Animals

Two clones of *D. pulex* were used in this study. Clone TCO was isolated from Slimy-Log pond near the Pacific coast in Oregon, USA (GPS coordinates N 43.830013, W -124.148152). TCO was chosen for genome sequencing (Colbourne *et al.* 2011). Clone Gerstel was isolated from a pond in northern Germany (Koch *et al.* 2009). Both clones have been cultivated in stock cultures for several years. *D. pulex* were reared in 800 ml aged and aerated tap water with no more than 15 animals per glass and were transferred into fresh water every second day.

Fourth instar larvae of *Chaoborus obscuripes* were obtained from an internet pet shop (Interquaristik.de).

Food

The strain of the green algae *Chlamydomonas klinobasis* used in this work was originally isolated from Lake Constance. The alga was grown in 5 L batch cultures at 20 °C and continuous light (3 fluorescent lamps, photon flux density of 95 µmol * s^{-1} m^{-2}) in sterile Cyanophycea medium according to (von Elert & Juttner 1997) to which 1 ml * l^{-1} of a vitamin stock solution (modified according to (Guillard 1975) was added. Every second day, one litre of the culture was replaced by fresh sterile medium. Food algae were screened through a 30 µm mesh to remove larger algal agglomerations. *C. klinobasis* was used as *Daphnia* diet for all cultures prior to the experiment.

During the experiments, additionally the two algae *Scenedesmus obliquus* SAG 276-3a and *Cryptomonas sp.* SAG 26.80 were used that were cultivated under same conditions as *C. klinobasis*, except that culture medium for *Scenedesmus* cultures was not supplemented with vitamins. Four days prior to the experiments, the animals were transferred to the respective algal food. *Cryptomonas sp*. is known to contain high amounts of eicosapentaenoic acid (von Elert & Stampfl 2000).

To exclude possible macro-nutritional limitation effects, C:N:P ratios of all three algal foods were determined during the experiment in triplicates according to (Moelzner & Fink 2014).

Growth experiments

Growth experiments with both clones on all three algal food treatments (*Scenedesmus obliquus, Chlamydomonas klinobasis and Cryptomonas* sp.) were performed in order to test for differences in food quality as indicated by effects on life-history parameters. Egg bearing (third clutch of eggs) individual *Daphnia* females, which had been reared on the respective food for four days, were placed in individual beakers containing 100 ml of aged tap water and 2 mg POC/l algal food. Animals were transferred to fresh medium every day. After the hatching, the mothers were removed, and the initial dry weight of the neonates was determined. Subsequently, six juveniles were kept in the beakers until they reached maturity, indicated by freshly deposited eggs in the brood pouch. Dry weight was determined weighing three adult individuals. Somatic growth rates were calculated according to (Wacker & von Elert 2001) with the formula $g = [\ln(dw_t)-\ln(dw_0)] \times d^{-1}$ with the body dry weight of a subsample of the animals at the beginning (dw_0) and end (dw_t) of the experiment and with the length of the experiment in days (d). All treatments were replicated three-fold. The same setup was used to calculate population growth rates with the exception that animals were kept in culture until the release of the third clutch of eggs. Time until the release of each clutch was recorded, and eggs in the brood chambers of all experimental animals were counted. As according to (Brzezinski & von Elert 2007), population growth rates (r) were calculated from daily survival and fecundity of the first three clutches using Euler`s equation, $r = \Sigma\, l_x * m_x * e^{-rx}$, where l_x represents the age-specific survivorship, m_x the number of newborns on day x, and x the age in days. Further, time to maturity was recorded and compared.

Preparation of *Chaoborus* incubation water extract

Approximately 1000 *Chaoborus* larvae were incubated for 24 hours in one litre of aged tap water. The incubation water containing *Chaoborus* kairomones was filtered through glass fibre filters (MN 85/220 pore size approximately 0.4 µm, Macherey & Nagel, Düren, Germany) and methanol was added to obtain a concentration of 1 %. For bulk enrichment of the kairomones, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 ml, end-capped, Varian Mega Bond Elut, Agilent Technologies) was pre-conditioned with 50 ml 1 % methanol-water prior to passing 1 l of sample through the cartridge. The loaded cartridge was washed with 50 ml of ultrapure water containing 1 % methanol and then eluted with 50 ml of methanol. The methanolic eluates originating from 20 l of *Chaoborus* incubation water were pooled, evaporated to dryness using a rotary evaporator and redissolved in 1 ml of methanol.

Bioassay on neck-teeth induction

D. pulex mothers with their third clutch just being deposited into the brood pouch (yolk eggs) were used for all experiments. Neck-teeth induction has been shown to be maximized, when the kairomone exposure starts during the second stage of embryonic development (Naraki *et al.* 2013). Bioassays were conducted with one individual *Daphnia* mother per glass jars. Kairomone treatments contained 5 µl *Chaoborus* incubation water extract, which was evaporated to dryness before applying 100 ml aged tap water to the jar. Control jars contained 5 µl methanol as negative control. Mothers as well as juveniles were fed above the ILL with the respective food (*Scenedesmus obliquus*, *Chlamydomonas klinobasis* or *Cryptomonas* sp.). The experiment was performed with five replicates, except for the *D. pulex* clone Gerstel *Chlamydomonas* treatment, which was replicated threefold. Immediately after hatching, the mothers were removed and the neonates monitored hourly for moulting, indicated by exuviae in the jars, until the juveniles reached the third larval instar. Neck-teeth development was quantified in the second larval instar, using a scoring system introduced by (Tollrian 1993).

The second larval instar is also know to produce the most pronounced neck-teeth (Tollrian 1993).

Electron microscopy

We used a Scanning Electron microscope (FEI Quanta 250) that was operated in the ESEM mode (Environmental Scanning Electron Microscope) to take the electron micrographs depicted in (Fig. 1).

Preparation of liposomes

Liposome stock suspensions were prepared according to (von Elert *et al.* 2012).

Food supplementation experiments

Four days prior to the experiments *D. Pulex* mothers precultured on *C. klinobasis* with their first clutch of eggs deposited in the brood pouch were transferred to either *Scenedesmus obliquus* or *Cryptomonas* sp. as food. Additionally, a mixed food treatment with 50 % of the carbon derived from *S. obliquus* and 50 % of the carbon derived from *Cryptomonas* sp. as well as supplementation treatments with 2 mg C * l^{-1} *S. obliquus* supplemented with 160 µl of a liposome stock suspension per litre was carried out. The liposomes contained either no additional fatty acids (control liposomes), or vaccenic acid (VCA), arachidonic acid (ARA), or eicosapentaenoic acid (EPA). When the animals had matured and deposited their third clutch of eggs in the brood pouches, a bioassay on neck-teeth induction was carried out as described above, replicated five-fold.

In a second food supplementation experiment, *Daphnia* neonates were again reared on *C. klinobasis* until the deposition of the first clutch. They were then transferred to medium containing 2 mg C * l^{-1} of *S. obliquus*. In order to investigate the influence of maternal effects on the strength of neck-teeth induction with regard to food quality, these mothers released their third clutch of eggs either into medium containing only 2 mg C * l^{-1} of *S. obliquus* or *S. obliquus* supplemented with 160 µl of either control or EPA liposomes per litre. All three

treatments contained *Chaoborus* kairomones and were repeated six-fold according to the bioassay protocol described above.

Statistics

All statistical calculations were performed with Sigma Plot 11 (Systat Software Inc.). All data were checked for homoscedasticity (equal variance test). If datasets consisted of only two groups, a subsequent t-test was performed. If there were more than two groups, a one way ANOVA was calculated, followed by a Tukey HSD, if only kairomone treatments were compared. If there were two grouping factors (food and kairomone) a two-way ANOVA followed by a Tukey HSD was calculated. For the correlation of the parameters 'neck-teeth score' and 'time needed to reach larval instar three', a linear regression analysis was performed, if normality tests and constant variance tests had been passed. The significance level was set to $p < 0.05$ for all tests.

Results

Two clones of *D. pulex* were cultivated on three different food algae and were, immediately after deposition of the third clutch, transferred to water with or without *Chaoborus* kairomone. Due to the molar C:N:P ratios of all three algae (*Chlamydomonas*: 111:19:1; *Scenedesmus* 123:18:1; *Cryptomonas* 81:16:1) stoichiometric food quality constraints for *Daphnia* could be excluded (Urabe & Watanabe 1992). As an indicator for food quality, somatic and population growth rates of both clones were determined in separate growth experiments, on which the subsequent kairomone bioassays were based. Further, time to maturity was recorded. Thus, no data on growth of *D. pulex* on *Scenedesmus* are available, since no animals from the *Scenedesmus* food treatment reached maturity. No significant differences were found for somatic growth rates of animals reared on *Chlamydomonas* or *Cryptomonas* for either clone (clone TCO on *Chlamydomonas* 0.68 * d^{-1} and on *Cryptomonas* 0.58 * d^{-1}; clone Gerstel on *Chlamydomonas* 0.65 * d^{-1} and on *Cryptomonas* 0.65 * d^{-1}) (t-test for TCO: $t(4) = 2.196$, $p = 0.093$; t-test for Gerstel $t(4) = 0.140$, $p = 0.896$). However, both clone TCO and clone Gerstel, exhibited significant differences in time to maturity and in population growth rate on the different food algae. Time to maturity was significantly shorter for animals from the *Cryptomonas* treatments (3.75 days for clone TCO and 3.625 days for clone Gerstel) compared to animals from the *Chlamydomonas* treatments (4.75 days for clone TCO and 4.5 days for Gerstel) (t-test for TCO: $t(14) = 4.320$, $p < 0.001$; t-test for Gerstel $t(14) = 3.326$, $p = 0.005$).

Further, population growth rates differed in an equal manner. Population growth was higher for animals from the *Cryptomonas* treatment (0.51 * d^{-1} for clone TCO and 0.511 * d^{-1} for clone Gerstel) compared to animals from the *Chlamydomonas* treatment (0.41 per day for clone TCO and 0.40 for clone Gerstel) (t-test for TCO: $t(14) = 6.022$, $p < 0.001$; t-test for Gerstel $t(14) = -8.258$, $p < 0.001$).

Kairomone bioassay

Neonates were examined for neck-teeth in the second larval instar, and the time was recorded until the juveniles reached the third larval instar. Juveniles of *D. pulex* clone TCO (Fig. 2 a) in the second larval instar showed a strong kairomone induced neck-teeth induction in the *Scenedesmus* treatment (83 %). Juveniles from the *Chlamydomonas* treatment showed a significantly reduced neck-teeth induction when exposed to kairomone (14 %) (t-test: $t = 18.427$, $p < 0.001$). Animals from the *Cryptomonas* treatment did not show any neck-teeth induction at all (0 %). Further, there was no neck-teeth induction throughout all control treatments, irrespective of the algal food (0 %).

Juveniles of *D. pulex* clone Gerstel (Fig. 2 b) exhibited neck-teeth induction throughout all food treatments, with some induced morphs even in the control treatments. A two-way ANOVA showed statistically significant differences for the factors food ($p < 0.001$, $F_{2, 20} = 62.014$), kairomone ($p < 0.001$, $F_{1, 20} = 1506.406$) as well as for the interaction of food x kairomone ($p < 0.001$, $F_{2, 20} = 49.755$). Neck-teeth induction was analyzed using a Tukey`s HSD, revealing a higher induction in the kairomone treatment than in the respective control (for all three food treatments $p < 0.001$), with neck-teeth inductions in all controls below 3.5 %. No statistical differences were found between the control treatments (Tukey`s HSD: *Scenedesmus* vs. *Chlamydomonas* $p = 0.998$; *Scenedesmus* vs. *Cryptomonas* $p = 0.746$; *Chlamydomonas* vs. *Cryptomonas* $p = 0.772$). Further, the strength of neck-teeth development in the presence of kairomone did not differ between the *Scenedesmus* (81 %) and the *Chlamydomonas* treatment (74 %) (Tukey`s HSD: $p = 0.062$). Solely juveniles of the *Cryptomonas* treatment with kairomone had a significantly lower neck-teeth induction of 44 % (Tukey`s HSD *Cryptomonas* vs. *Scenedesmus* $p < 0.001$; *Cryptomonas* vs. *Chlamydomonas* $p < 0.001$).

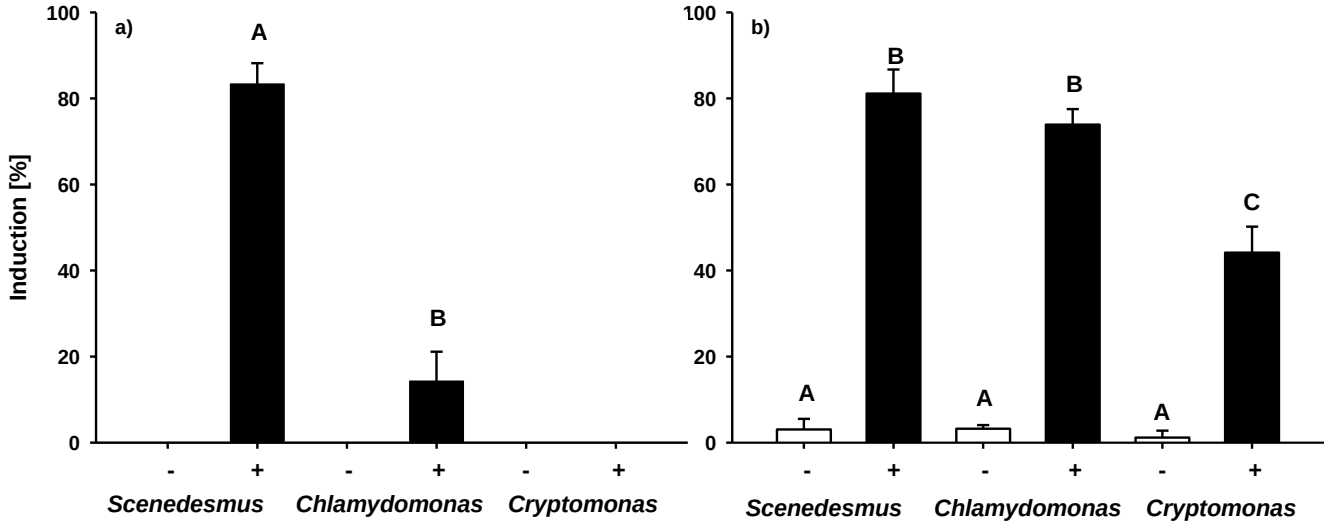

Figure 2: Mean (n = 5, + SD, except for clone Gerstel on *Chlamydomonas* n = 3, + SD) neck-teeth inductions of second instar juveniles of *D. pulex* clone TCO (a) and clone Gerstel (b) in the absence (-) or presence of *Chaoborus* kairomone. Animals were grown either on *Scenedesmus obliquus* (*Scenedesmus*), *Chlamydomonas klinobasis (Chlamydomonas)* or *Cryptomonas* sp. (*Cryptomonas*) food. Different characters indicate significantly different groups within each clone ($p < 0.05$).

Time to third larval instar

Determination of the time to reach the third larval instar was based on hourly examinations of experimental jars for exuviae. For clone TCO (Fig. 3 a), a two-way ANOVA revealed significant differences for the factor food ($p < 0.001$; $F_{2, 22} = 318.948$). No differences were found for the factor kairomone ($p = 0.005$; $F_{1, 22} = 4.283$) nor for the interaction of both factors (food x kairomone: $p = 0.764$; $F_{2, 22} = 0.272$). A Tukey`s HSD was used to test for differences between the different food treatments. Animals from the *Scenedesmus* treatment needed the longest time to reach the 3rd larval instar (72 h for control and 74 h for kairomone, Tukey`s HSD: $p < 0.001$ for *S. obliquus* versus *C. klinobasis* and for *S. obliquus* versus *Cryptomonas* sp.), followed by the animals in the *Chlamydomonas* treatment (51 h for control and 53 h for kairomone, Tukey`s HSD: $p \leq 0.001$ for *Chlamydomonas* versus *Scenedesmus* and $p = 0.001$ *Chlamydomonas* versus *Cryptomonas*). Animals from the *Cryptomonas*

treatment reached the third larval instar fastest (47 h for control and 48 h for kairomone, Tukey`s HSD: $p \leq 0.001$ for *Cryptomonas* versus *Scenedesmus* and $p = 0.001$ for *Cryptomonas* versus *Chlamydomonas*).

For clone Gerstel (Fig. 3 b), a two-way ANOVA revealed no significant effects of the factor food ($p = 0.065$; $F_{2, 15} = 3.304$), kairomone ($p = 0.757$; $F_{1, 15} = 0.099$) or their interaction (food x kairomone $p = 0.175$; $F_{2, 15} = 1.962$).

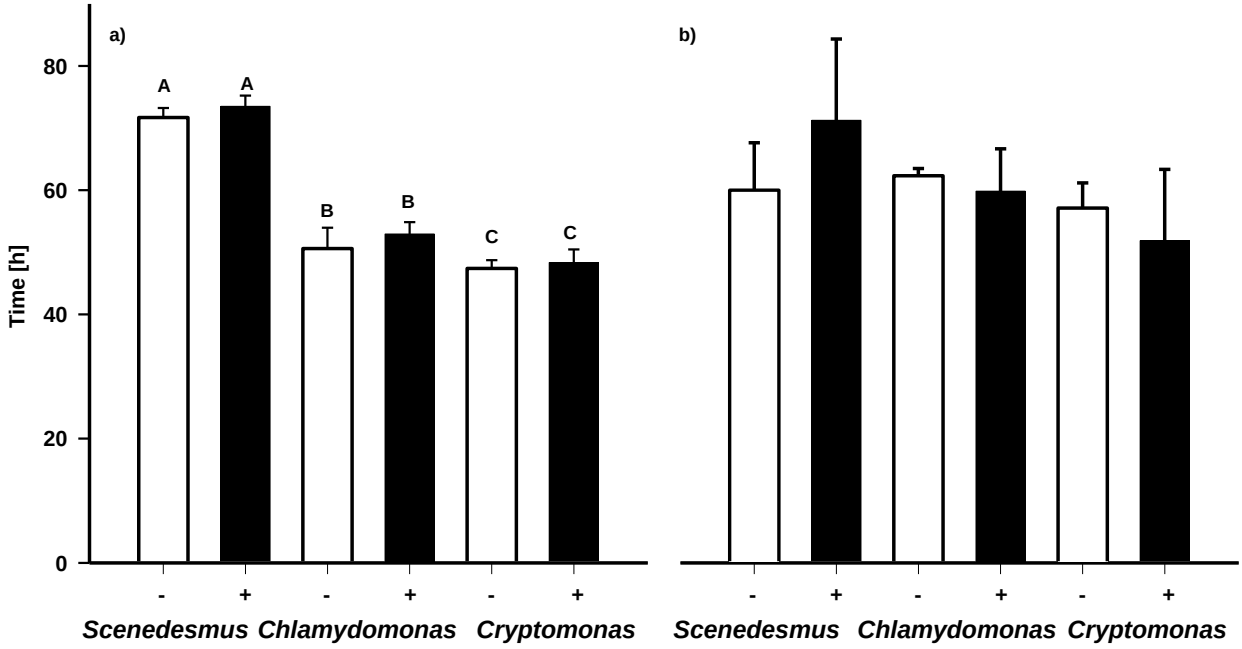

Figure 3: Mean (n = 5, + SD) time for neonates of *D. pulex* clone TCO (a) and clone Gerstel (b) from hatching until moulting to the third larval instar in the absence (-) and presence (+) of *Chaoborus* kairomone. Animals were grown either on *Scenedesmus obliquus* (*Scenedesmus*), *Chlamydomonas klinobasis (Chlamydomonas)* or *Cryptomonas* sp. (*Cryptomonas*) food. Different characters indicate significantly different groups within each clone ($p < 0.05$).

Neck-teeth induction as a function of developmental time

When the degree of neck-teeth induction in the second larval instar of the *Chaoborus* kairomone treatments was depicted as a function of the developmental time of the juveniles to reach the third larval instar, a regression analysis revealed a significant linear correlation

between both parameters for clone TCO (Fig 4 a, $F_{2, 9} = 214.49$, $p < 0.0001$, $r^2 = 0.95$). A normal distribution of the data was given (Shapiro-Wilk normality test: $p = 0.7587$). A longer time until the third instar went along with a stronger neck-teeth induction in the second larval instar in the presence of identical concentrations of *Chaoborus* kairomone. For clone Gerstel (Fig 4 b), no significant linear correlation could be shown (regression analyses: $F_{2, 6} = 1.77$, $p = 0.22$, $r^2 = 0.20$) although the trend indicates a similar relation between those two parameters. However, a normal distribution of the data was given (Shapiro-Wilk normality test: $p = 0.3453$).

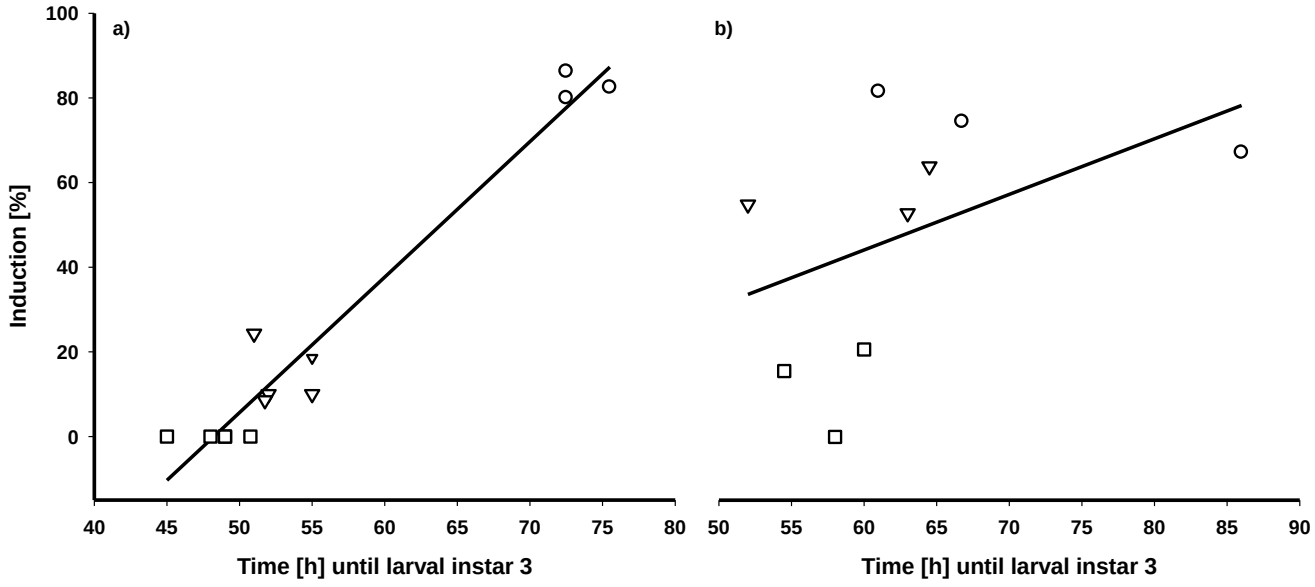

Figure 4: Neck-teeth induction by *Chaoborus* kairomone as a function of developmental time from hatching until the moulting to the third larval instar for *D. pulex* clone TCO (a) and clone Gerstel (b). Datapoints represent single replicates for *D. pulex* fed on *Scenedesmus obliquus* (circles), *Chlamydomonas klinobasis* (triangles) and *Cryptomonas* sp. (squares) r^2 values for the regressions lines are 0.95 (TCO) and 0.2 (Gerstel).

Causes for food quality effects on neck-teeth induction

In order to test if the differences in food quality are caused by differences in fatty acid content of the food algae, low quality food (*S. obliquus*) was supplemented either with liposomes loaded with different fatty acids or with high quality food (*Cryptomonas* sp.) and provided as

diet for *D. pulex* clone Gerstel. Diets of *Cryptomonas* or *Scenedesmus* only served as control treatments for high and low neck-teeth development. In the absence of *Chaoborus* kairomone, neck-teeth induction always was < 5 %, which was in line with negative controls of the previous experiment. Thus only kairomone treatments are depicted (Fig. 5) and subsequently used for statistical analyses.

As in the previous experiment, juveniles from mothers from the *Scenedesmus* treatment showed a higher neck-teeth induction (75 %) than juveniles from mothers from the *Cryptomonas* treatment (44 %) (one-way ANOVA $F_{5, 28} = 28.844$; $p < 0.001$ followed by a Tukey`s HSD $p < 0.001$). Neck-teeth induction from the *Scenedesmus* treatments supplemented with control liposomes (74 %), with liposomes containing the monounsaturated vaccenic acid (69 %) or with liposomes containing the polyunsaturated arachidonic acid (70 %) did not differ significantly from the *Scenedesmus* control treatment (Tukey`s HSD: *Scenedesmus* vs. control liposomes $p = 1.0$; *Scenedesmus* vs. vaccenic acid $p = 0.663$; *Scenedesmus* vs. arachidonic acid $p = 0.834$), which indicated that neither low concentrations of vaccenic nor of arachidonic acid in *S. obliquus* caused the high neck-teeth induction observed in *D. pulex* feeding on this alga .

On the other hand, neck-teeth induction in juveniles from mothers from the *Scenedesmus* treatment supplemented with liposomes containing eicosapentaenoic acid (43 %) did not differ from the *Cryptomonas* control treatment (Tukey`s HSD $p = 0.999$). Also, juveniles from mothers fed with a mixed diet of *Cryptomonas* and *Scenedesmus* (45 %) did not differ from the *Cryptomonas* control (Tukey`s HSD $p = 1.0$), which demonstrates that a low content of eicosapentaenoic acid in *S. obliquus* caused the high neck-teeth induction in *D. pulex* feeding on this alga.

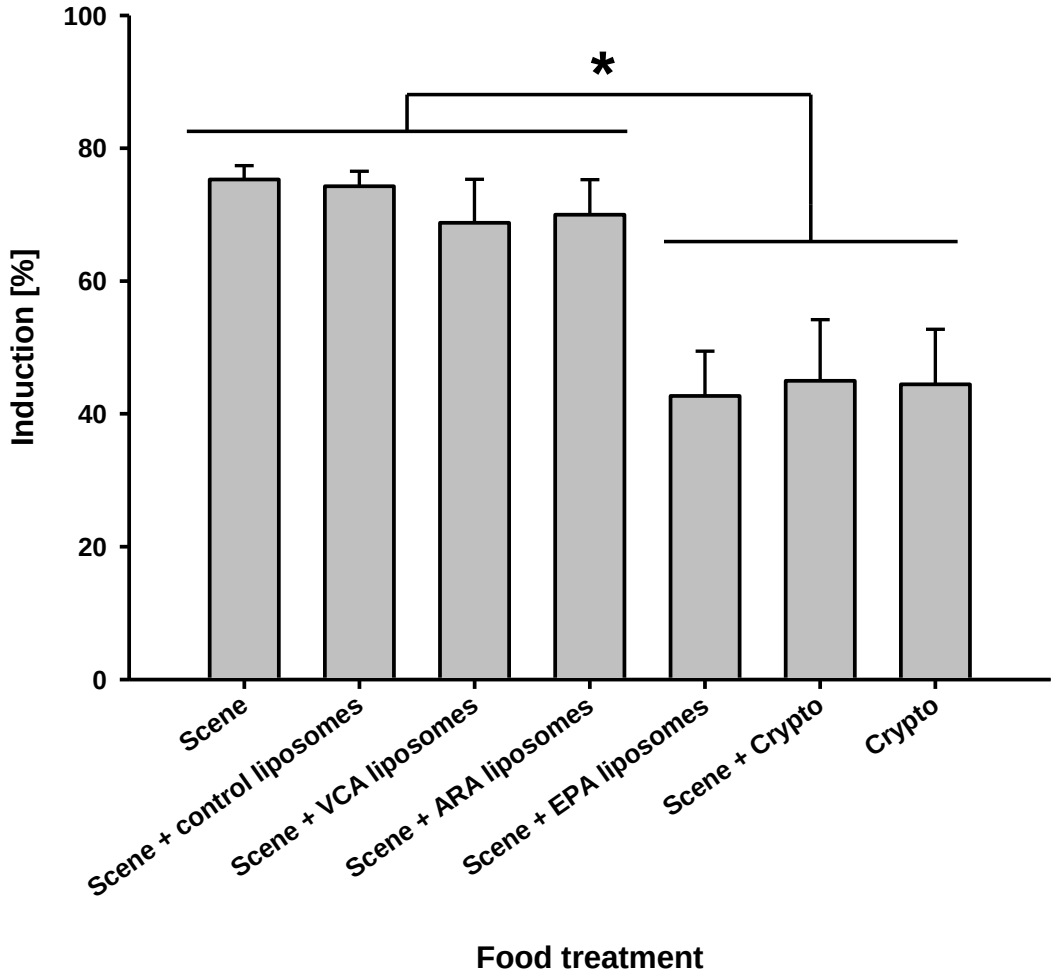

Figure 5: Mean (n = 5, + SD) neck-teeth inductions of second instar juveniles of *D. pulex* clone Gerstel exposed to *Chaoborus* kairomone. Mothers and neonates were fed with *Scenedesmus obliquus* (Scene) or *Cryptomonas* sp. (Crypto) either individually, equally mixed (Scene + Crypto) or supplemented with different liposomes. Control liposomes contained no additional fatty acids. Other liposomes contained either vaccenic acid (VCA), arachidonic acid (ARA) or eicosapentaenoic acid (EPA). * indicate significantly different groups ($p < 0.05$).

In order to test for maternal effects, mothers reared on *S. obliquus* released their offspring into medium containing differently favourable supplementations with liposomes (Fig. 6). A one-way ANOVA revealed no statistically significant differences ($F_{2, 15} = 3.641$; $p = 0.51$), demonstrating that the food consumed by the juveniles did not have an impact on the strength

of neck-teeth induction in the second juvenile instar, which strongly suggests that the maternal food quality and not that experienced by the juveniles affects the strength of neck-teeth induction.

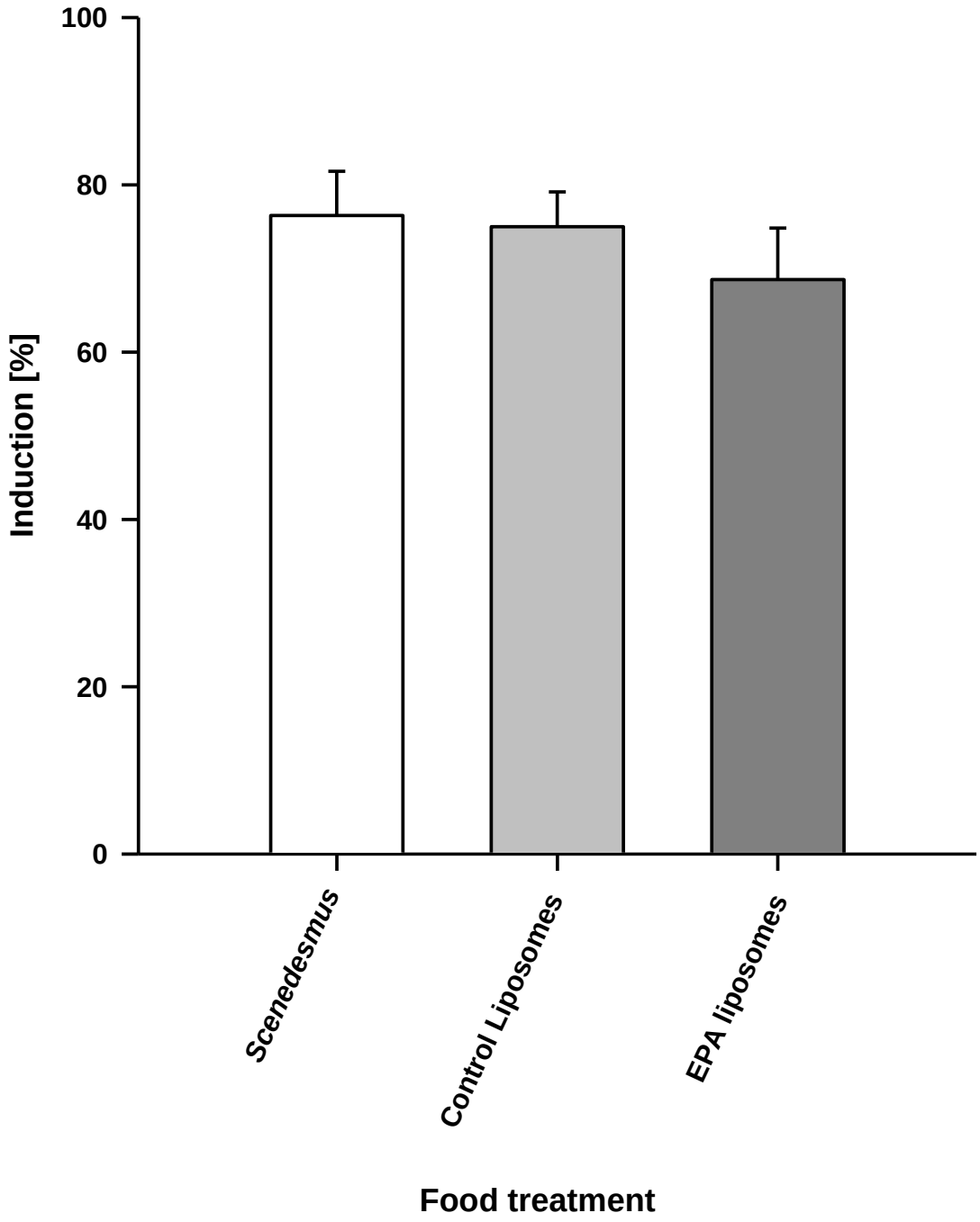

Figure 6: Mean (n = 6, + SD) neck-teeth inductions of second instar juveniles of *D. pulex* clone Gerstel derived from mothers fed with *Scenedesmus obliquus* exposed to *Chaoborus* kairomone and fed with *S. obliquus* (*Scenedesmus*) or *S. obliquus* supplemented with either control liposomes or liposomes containing eicosapentaenoic acid (EPA).

Discussion

In this study, a strong interaction of both predator kairomone and food quality on the strength of neck-teeth development in *D. pulex* could be demonstrated. The presence of *Chaoborus* kairomone induced differently strong neck-teeth developments dependent on the *Daphnia* genotype and on the diet the *Daphnia* were reared on. Many earlier studies have reported life-history changes induced by *Chaoborus* kairomone, with *Daphnia* needing a longer time to reach maturity (Tollrian 1995), a larger size at birth, a reduced juvenile growth rate, a delayed reproduction with fewer neonates in the first clutch and also a slight tendency towards an increased size at maturity (Riessen 1999; Riessen 2012). Here we focussed on the time to reach larval instar three, since the first two instars are the most vulnerable ones with respect to predation by larvae of *Chaoborus* (Swift & Fedorenko 1975). Hence, developmental time to larval instar three assesses the developmental time window that juvenile *D. pulex* are potential prey for larvae of *Chaoborus*, and no kairomone effects on this developmental time were observed. However, food dependent differences in time to larval instar three were found at least for clone TCO with *Daphnia* fed with *Scenedesmus* taking longest, *Chlamydomonas* being intermediate and *Cryptomonas* being fastest in reaching instar three. This was in line with the growth experiments, which showed that *Cryptomonas* sp. was of highest, *C. klinobasis* was of intermediate and *S. obliquus* was of low food quality with no individual *Daphnia* reaching maturity. Also, population growth rates of both clones showed the same pattern of algal food quality, which confirms the findings of (Lampert & Trubetskova 1996), that the somatic growth rate of *Daphnia* is a good predictor of the population growth rate. However, it should be noted that here we manipulated food quality whereas in (Lampert & Trubetskova 1996) food quantity had been varied experimentally .

Feeding on *Scenedesmus* resulted in a 57 % extended developmental time window compared to feeding on *Cryptomonas*, so that feeding on *Scenedesmus* would translate into a substantially increased risk for being preyed upon by larvae of *Chaoborus*. In line with this

we here report a strong positive correlation between strength in neck-teeth induction and time to reach larval instar three. This finding strongly suggests that the increased morphological defence counterbalances the higher predation risk a juvenile *D. pulex* has to face, when more time is needed to outgrow the critical body size, below which it is a suitable prey for larvae of *Chaoborus*.

Although the costs of neck-teeth formation are hard to quantify, (Riessen & Sprules 1990;Riessen 2012) calculated an 8-9 % population growth reduction for neck-teeth development in a responsive *D. pulex* clone induced by *Chaoborus* kairomone. In line with this we find that high quality food (here: *Cryptomonas* sp.) and thus high growth rates and short developmental times lead to reduced morphological defences and thus to reduced costs associated with morphological defence, which seems adaptive due to a lower encounter probability of juvenile *D. pulex* with larvae of *Chaoborus*.

In *D. pulex* clone Gerstel no effect of diet on the developmental time to the third larval instar was detected. Still, the same tendency as in clone TCO with neck-teeth induction decreasing with increasing food quality could be observed for the *Chaoborus* treatment. As a result no significant correlation between the time needed to reach the third larval instar and the strength of neck-teeth induction was found, although a positive tendency could be also observed. Compared to clone TCO, neck-teeth induction was generally higher in clone Gerstel, with neck-teeth even developing in the control treatment in the absence of kairomone. Thus, in clone Gerstel neckteeth are to a certain degree constitutive, and on top of this, can be induced by kairomone to an even higher level than in TCO. The reason for this might lie in the different geographical origins of the two clones. As nothing is known about their ecological background, we can only speculate that the higher level of constitutive neck-teeth deployment in clone Gerstel and its generally higher neck-teeth induction might reflect a rather constant predation risk by larvae of *Chaoborus*.

In this work, the supplementation of *S. obliquus* with eicosapentaenoic acid (EPA) has been shown to suppress neck-teeth development in *D. pulex*. Additional EPA is the most reasonable explanation for a similar suppression of neck-teeth development when *S. obliquus* was supplemented with *Cryptomonas* sp., as *Cryptomonas* sp. contains high concentrations of EPA (von Elert & Stampfl 2000), which is absent from *S. obliquus* (von Elert 2002). The observed suppression of neck-teeth development was specific for the n-3 polyunsaturated fatty acid (n-3 PUFA) EPA, as this effect was neither observed with the n-6 PUFA arachidonic acid (ARA) nor with the monounsaturated vaccenic acid. Using the same strain of *S. obliquus* (von Elert 2002) showed that supplementation with ARA had no effect on growth of *Daphnia*, but addition of EPA and other n-3 PUFAs led to an increase of juvenile growth in *Daphnia*. As we have applied no other n-3 PUFA than EPA it currently remains unclear if the observed suppression of neck-teeth development is specific for n-3 PUFAs in general or for EPA only. As juvenile growth of *Daphnia* on *S. obliquus* is limited by the absence of EPA ((von Elert 2002), (Becker & Boersma 2003)), it is reasonable to assume that as well in our experiments supplementation with EPA resulted in increased growth of juvenile *D. pulex*. Thus it remains to be tested if the suppression of neck-teeth development by EPA is a compound-specific effect or fairly the effect of enhanced growth. In the latter case elevated ambient temperatures should similar to EPA result in suppressed neck-teeth development. Likewise enhanced neck-teeth development might be caused by heavy metals, herbicides or parasites that have been shown to negatively affect somatic growth in *Daphnia* ((Kashian & Dodson 2002), (Zeman *et al.* 2008), (Engelbrecht *et al.* 2013)).

The concentration of EPA in natural phytoplankton is a powerful predictor of *Daphnia* growth within a lake (Müller-Navarra 1995; Wacker & von Elert 2001; Müller-Navarra *et al.* 2000) and among lakes (Müller-Navarra *et al.* 2004), which suggests that this PUFA is a limiting biochemical constituent of the natural diet, in particular in eutrophic lakes (Persson *et al.* 2007). These evidences for the significance of temporarily strong EPA-limitation of

Daphnia in nature suggest that for a given kairomone concentration the resultant degree of neck-teeth induction will be highly coupled to the seasonal variations of EPA in the edible fraction of the natural phytoplankton.

Further it could be shown that the effects of different food qualities refer to the maternal diet and not to that of the offspring. This was in line with the findings of (Bennett & Murray 2014), who found indirect evidence for a putative role of maternal food for the strength of morphological defence of the offspring by positively relating the increase in relative tail depth of tadpoles as a response to predatory dragonfly larvae with maternal fitness. In a kairomone bioassay, neck-teeth induction in the second juvenile instar was not suppressed in juveniles from mothers reared on food of low quality, when the low food was supplemented with EPA liposomes after hatching. This is in line with the results of (Demott & Müller-Navarra 1997), and (Sperfeld & Wacker 2012), who both reported that the negative effects of low food quality on juvenile growth could, at least partly, be mitigated if the maternal diet had been of higher quality.

A major determinant of predation risk is the encounter probability of predator and prey. In line with the fact that this probability is, among others, density dependent, inducible anti-predator defences have been shown to increase with kairomone concentration (von Elert & Pohnert 2000; von Elert & Stibor 2006; Tollrian 1993). Here, in a system with a gape-limited predator, the overall encounter probability further depends on developmental time, as this determines the width of the time window that a prey remains vulnerable. To our knowledge this is the first case that demonstrates that inducible anti-predator defences are modulated by the food quality of the prey. These novel findings suggest that food quality can constrain inducible morphological defences, ultimately influencing the capacity of populations to utilize inducible anti-predator defences.

Acknowledgments

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Chapter 2

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Phenotypic plasticity in three *Daphnia* genotypes in response to predator kairomone: no evidence for juvenile hormone signalling but for involvement of chitin deacetylases.

Abstract

The genetic background of inducible morphological defences in *Daphnia* is still largely unknown. In a target gene approach, by applying a gradient of three *Daphnia* genotypes with increasing strength of neck-teeth induction, we report a high correlation of neck-teeth induction in *D. pulex* and relative gene expression of two chitin deacetylases. Further, previous studies suggested genes from both the juvenoid and the insulin hormone signalling pathways as well as several morphogenetic genes downstream to be responsible for the neck-teeth induction in *Daphnia pulex*. However, these data on previously suggested genes reported were not supported by this study. None of the three *D. pulex* clones did show an upregulation of these five genes as a response to predator kairomone, which is interpreted as the result of refined methods used for both RNA sampling and kairomone enrichment, which yielded unambiguous results compared to earlier studies.

Introduction

Defences against predation are a crucial issue for organisms throughout ecosystems and can be divided into two types. Constitutive defences are deployed independent of any cue that indicates the presence of a predator and are thus expressed regardless whether there is a threat of predation or not. Theory predicts constitutive rather than inducible defences in the presence of a fairly constant risk of predation or if the defence does not impose costs (Stearns 1993).

On the other hand, inducible defences are a means of prey organisms to respond to a varying risk of predation. Inducible defences are triggered by the perception of the predator through predator associated cues, which, in freshwater ecosystems, are often waterborne chemicals, so called kairomones. Kairomone induced defences provide a fitness advantage to the receiver while providing a fitness disadvantage to the emitter (Dicke & Sabelis 1988). Inducible defences should be adaptive, if predation pressure is unpredictable and if the defence is associated with high costs (Harvell 1990).

Daphnia, a keystone species and model organism for freshwater ecosystems, has been shown to respond to the presence of predators by a shift in life history, a change in behavior or a modification of morphology (von Elert 2012). *Daphnia* feeds unselectively on phytoplankton and thus links higher trophic levels to primary production, being preyed upon by both vertebrates like fish and a variety of invertebrates. Very important invertebrate predators of *Daphnia* are aquatic larvae of the phantom midge *Chaoborus* sp., which are characterized by a cosmopolitan distribution (Borkent 1981) and which have been shown to reach high abundances (e.g. (Wissel *et al.* 2003) and (Voss & Mumm 1999). Therefore the interaction of *Daphnia* and *Chaoborus* has been studied in great detail. For *D. pulex* it has been shown that neonates have a larger size at birth, when exposed to *Chaoborus* kairomone during embryonic development, which should be advantageous, since *Chaoborus* is a gape limited predator, with a strike efficiency that is lower at lager prey sizes (Riessen & Trevett-Smith 2009). Further, *D. pulex* migrates upwards in the water column as a response to *Chaoborus*

kairomone ((Boeing *et al.* 2006) and (Oram & Spitze 2013)). Finally, in the presence of kairomone from *Chaoborus* larvae, juveniles of *D. pulex* develop neck-teeth at the back of the head, a morphological defence that has been shown to reduce mortality due to *Chaoborus* predation (Havel & Dodson 1984). In line with this, induction of neck-teeth occurs only in those instars, which are vulnerable to the gape-limited predation by *Chaoborus* sp. (Riessen & Trevett-Smith 2009).

Although being a thoroughly studied topic, the regulation of the induction of neck-teeth on the level of hormones remains poorly understood. However, the deciphering of the underlying endocrinology poses a task that is hard to accomplish, since quantification of hormone titres by analytical and/or immunological approaches in small animals as *Daphnia* sp. is rather challenging. Thus molecular approaches have been used to investigate the endocrinology of morphological defences in response to kairomones from larvae of *Chaoborus*.

The juvenile hormone pathway offered a promising perspective, since it has been shown to be involved in the regulation of moulting in crustaceans (Chang *et al.* 1993) and thus is putatively connected to predator induced changes in life history. Further, (Olmstead & LeBlanc 2002) demonstrated, that the juvenoid hormone methyl farnesoate was capable of inducing male formation in *Daphnia* embryos and thus was involved in the induction of a morphologically distinct *Daphnia* phenotype. Hence, it seemed plausible to assume a potential involvement of the juvenile hormone pathway in the induction of neck-teeth as a response to predator kairomones.

In line with this differential gene expression in *D. pulex*, either in genes involved in synthesis/degradation of juvenile hormones (Miyakawa and others 2010) or in a network of nuclear receptors that were assumed to be specific for juvenoid endocrinology (Dennis and others 2014) was investigated in the absence or presence of extracts from larvae of *Chaoborus* sp. Unfortunately in neither study has neckteeth-induction been monitored, so that changes in gene expression cannot be unambiguously attributed to an increase in neck-teeth induction.

However, identifying the genes responsible for neck-teeth induction is of high interest in order to obtain a deeper understanding of the complexity of predator-prey interactions of *Daphnia* and *Chaoborus*.

On the other hand, *D. pulex* is not the only *Daphnia* species known to exhibit inducible morphological defences as a response to the presence of predator kairomones. *Daphnia magna*, for example, has been shown to develop an increased bulkiness when directly exposed to *Triops cancriformis* or its chemical cues, which provides a protection against predation (Rabus & Laforsch 2011). Exposure of *T. cancriformis* kairomone has been shown to lead to upregulation of two chitin deacetylases (1 and 2A) on protein level (Otte *et al.* 2014) which catalyze N-deacetylation of chitin. In *Tribolium castaneum*, several types of chitin deacetylases have been identified in the exoskeletal epidermis (Arakane *et al.* 2009), which allows the conclusion that these enzymes are also involved in exoskeletal development and thus morphological defence induction in *Daphnia*. These findings provide another approach to elucidate the involved network of responsible genes by providing candidate genes beyond hormonal pathways.

This study uses three different clones of *D. pulex*, which differ in responsiveness to *Chaoborus* kairomone, i.e. the clones express different intensities of neck-teeth induction at equal *Chaoborus* kairomone concentrations. With this "clonal-gradient", gene expression levels in the absence and presence of kairomone were determined in order to correlate the strength of gene expression to the neck-teeth induction throughout the clones. Therefore, gravid females of the three *D. pulex* clones were exposed to *Chaoborus* kairomone, and induction of neck-teeth in newbornes was quantified and the same newbornes were subsequently used for analysis of gene expression by qPCR. We determined the expression of five putative target genes from (Miyakawa *et al.* 2010) that had shown the most marked upregulation. We further hypothesized that two chitin deacetylases that, using proteomics, have been shown to be involved in another morphological defence in *D. magna* (Otte *et al.*

2014) would as well be involved in the morphological changes induced by larvae of *Chaoborus* in *D. pulex*. This study aims at providing a deeper understanding of the involvement a several previously proposed candidate genes by applying a more sophisticated sampling method as well as incorporating data on the strength of neck-teeth induction and directly correlating this to a clone specific gene-expression of those candidate genes. With this, it should be possible to differentiate between genes that are directly involved in neck-teeth induction and those, which are part of a more general stress response to the presence of predator kairomone.

Material and methods

Animals

Three clones of *Daphnia pulex* were used in this study. *D. pulex* clone TCO was isolated from a naturally inbred population inhabiting a permanent pond in the Siuslaw National Forest, near the Pacific coast in Oregon, USA. *D. pulex* clone TCO was chosen for genome sequencing (Colbourne *et al.* 2011). *D. pulex* clone Gerstel was isolated from a pond in northern Germany (Koch *et al.* 2009). *D. pulex* clone Münster was originally isolated from a pond in Münster, Gievenbeck. It was used in a study by (Kuster & von Elert 2013)

All clones have been cultivated in stock cultures for several years. All *D. pulex* were reared in 800 ml aged and aerated tap water with no more than 15 animals per glass with 2 mg POC / L of *Chlamydomonas klinobasis* as food and were transferred into fresh water and food every second day.

Forth instar larvae of *Chaoborus obscuripes* were obtained from an internet pet shop (Interquaristik.de).

Food

The strain of the green algae *Chlamydomonas klinobasis* used in this work was originally isolated from Lake Constance. The alga was cultured in 5 L batch cultures in glass bottles at constant 20 °C and continuous light conditions (3 fluorescent lamps with a photon flux density of 95 µmol*s^{-1} m^{-2})in sterile Cyanophyceae medium according to (von Elert & Juttner 1997) with vitamins (thiamine hydrochloride 300 nM, biotin 2 nM, and cyanocobalamine – vitamin B_{12} 0.4 nM. (modified according to (Guillard 1975)).

Preparation of *Chaoborus* incubation water extract

Approximately 1000 *Chaoborus* larvae were incubated for 24 hours in one litre of aged tap water. The incubation water containing *Chaoborus* kairomones was filtered through membrane filters (pore size: 0.45 µm). For bulk enrichment of the kairomones, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 ml, end-capped, Varian Mega Bond Elut, Agilent

Technologies) was pre-conditioned with 50 ml 1 % methanol-water prior to adding the sample. Methanol was added to the filtered *Chaoborus* incubation water to obtain a 1% concentration, and 1 l of sample was passed through the cartridge. The loaded cartridge was washed with 50 ml of ultrapure water with 1 % methanol and then eluted with 50 ml of methanol. The eluates originating from 20 l of *Chaoborus* incubation water were pooled, evaporated to dryness using a rotary evaporator and redissolved in 1 ml of methanol.

Bioassay

D. pulex mothers with their third clutch just deposited in the brood pouch (yolk eggs) were used for all experiments. Neck-teeth induction has been shown to be maximized, when the kairomone exposure starts during the second stage of embryonic development (Naraki *et al.* 2013). Bioassays were conducted in glass jars. Kairomone treatments contained 5 µl *Chaoborus* incubation water extract, which was evaporated to dryness before applying 100 ml aged tap water to the jars. Control jars contained 5 µl methanol as negative control. Within the first hour after hatching, the neck-teeth induction of the neonates was quantified using a scoring method introduced by (Tollrian 1993) , and animals were sampled for RNA extraction. Each treatment (*Chaoborus* incubation water and control) was carried out with 15 replicates for all three clones.

RNA extraction and reverse transcription

Neonates were stored at –80 °C immediately after hatching. Later, approximately fifty animals were pooled and RNA was extracted using the RNeasy extraction kit from QUIAGEN. Concentrations of RNA were determined immediately after extraction using a Nanodrop (Spectrophotometer ND-1000, software version 3.8.1, Thermo Scientific). RNA (1 mg) was reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA was stored at -20 °C.

qPCR

For normalization, two different endogenous controls (18S ribosomal RNA (18S) and 18S ribosomal RNA (28S)) were used in qPCR analysis. These endogenous controls were chosen from a given set of ten reference genes (Heckmann *et al.* 2006).

The candidate genes to be tested for response to *Chaoborus* kairomone were extradenticle (exd), escargot (esg), juvenile hormone acid methyltransferase (JHAMT), insulin-like receptor (InR) and tyramine beta-monooxygenase (TBM) as published by (Miyakawa *et al.* 2010), as well as the genes chitin deacetylase 1 and 2 (Cda1 and Cda2). Primers are given in Table 1.

Primers were designed with PRIMER 3 v. 2.3.5 and the quality checked with NETPRIMER (Premier Biosoft; Table 1). Melting curve analyses confirmed specific amplification without primer dimer formation. The data acquisition for the relative expression was performed on a 7300 qPCR system (Applied Biosystems). Each reaction contained 5 ng of cDNA template, 10 ml SYBR green PCR master mix and 2.5 mM of each primer in a final volume of 20 ml. Cycling parameters were 95 °C for 10 min for the initial start of the DNA polymerase, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s, and a final dissociation step with 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s and 95 °C for 15 s. The baseline and threshold for the cycle threshold (Ct) was set automatically, and each reaction was conducted in biological triplicates. Amplification efficiencies for every primer pair of each candidate gene were determined.

Table 1: Investigated candidate genes with respective primers and gene ID

Candidate gene (abbreviation)		Primer	*Daphnia pulex* gene ID
Chitin deacetylase 1 (Deacetylase 1)	F	TTCCCAACACTCCAGCTCTA	Dappu-309273
	R	GGAAGCGAGAAAAACGGTAAA	
Chitin deacetylase 2 (Deacetylase 2)	F	TTCTTCGTTTCGCACAAGTA	Dappu-327753
	R	TGCCATCGGTCCAGTATT	
escargot (esg)	F	CGATTGCCACAAGTCGTACT	Dappu-50534
	R	GCCCAGCGAGACGTAGAC	
extradenticle (exd)	F	GGGTAGTTCCTCGCCATACA	Dappu-219790
	R	ACCACCGTTGAGTCCCATAG	
Insulin-like receptor (InR)	F	GTGGAAAGCCAACGCACTA	Dappu-270048
	R	CGTTCCGTGGTCATCCTTAT	
Juvenile hormone acid methyl transferase (JHAMT)	F	CTTCGTTGACGGGCATAGAT	Dappu-300180
	R	CGAATCCATCGGGGAATACT	
Tyramine beta-monooxygenase (TBM)	F	TGGACCGTGACAATCACTACA	Dappu-1839
	R	GCGTTCTCCTTTTCCGTAGA	

Statistics

After qPCR, raw data were analysed with QBASEPLUS v. 2.0 (Biogazelle) based on QBASE (Hellemans *et al.* 2007) and GENORM (Vandesompele *et al.* 2002). Data on neck-teeth induction in the three *D. pulex* clones were checked for homogeneity of variances (equal variance test) and for normality (Shapiro–Wilk). An analysis of variance (one-way ANOVA) was carried out, followed by a Tukey's HSD multiple comparison test. Relative expression was equally checked for homogeneity of variances (equal variance test) and for normality (Shapiro–Wilk). T-tests were calculated for each gene pair separately. Data were subsequently subjected to a Bonferroni correction. For the correlation of gene expression level and neck-teeth induction, the data were checked for both constant variances and normality, and subsequently a linear regression analysis was carried out for each gene separately. A significance level of $p < 0.05$ was applied to all statistical analyses. All statistics were performed with SIGMAPLOT v. 11.0 (Systat Software).

Results

Neck-teeth induction of all clones

Neck-teeth inductions in the first juvenile instar of three differently inducible *D. pulex* clones, exposed to *Chaoborus* kairomone or control treatments without kairomone, were compared (Fig. 1). A one-way ANOVA revealed significant differences between the treatments ($F_{5, 84} = 519.324$; $p < 0.001$). Equal variances ($p = 0.387$) and normality ($p = 0.302$) of the data were given. Animals in the *D. pulex* TCO control treatment did not develop any neck-teeth at all and were thus excluded from all statistical analyses. A Tukey`s HSD revealed neck-teeth induction to be significantly higher in *D. pulex* Münster exposed to kairomone (88 % induction) when compared to all other treatments (Münster with kairomone versus all other treatments: $p < 0.001$). Juveniles from the *D. pulex* Münster control treatment had the second highest neck-teeth induction among all treatments (49 % induction) and were also significantly different from all other treatments (Tukey`s HSD $p < 0.001$ for Münster control versus all other treatments). The neck-teeth induction in the kairomone treatment of the clone Gerstel showed the third highest neck-teeth induction (20 % induction) and was also significantly different from all other treatments (Tukey`s HSD $p < 0.001$ for Gerstel with kairomone versus all other treatments). The treatments of clone Gerstel without kairomone (7 % induction) and clone TCO with kairomone (11 % induction) did not differ from one another (Tukey`s HSD $p = 0.425$) but both differed from all other treatments (Tukey`s HSD $p < 0.001$ for all other pairwise comparisons of clone TCO with kairomone and clone Gerstel without kairomone).

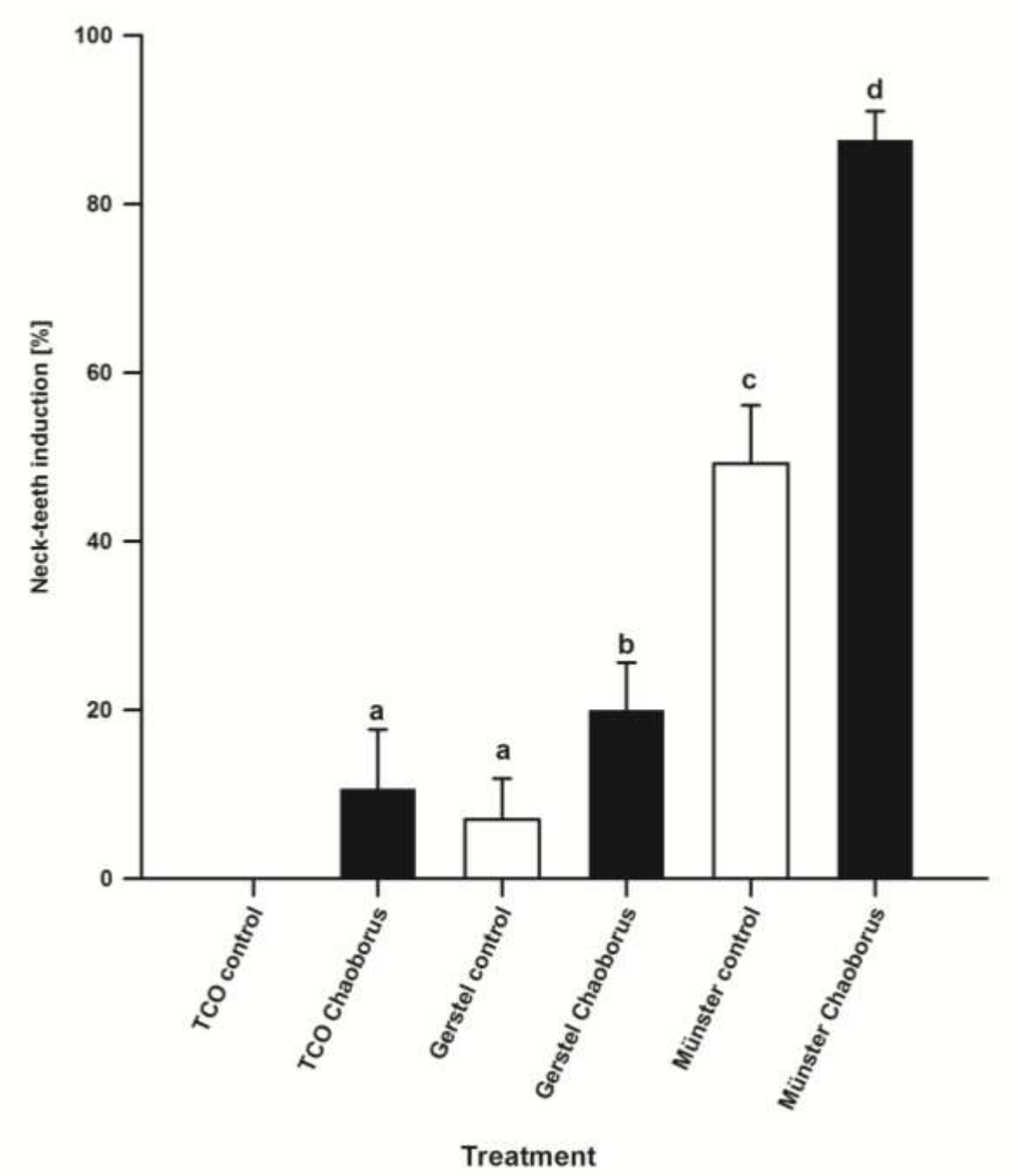

Figure 1: Mean (n = 15, + SD) neck-teeth inductions of first instar juveniles of *D. pulex* clone TCO, Gerstel and Münster in the presence (black bars) or absence (white bars) of *Chaoborus* kairomone. Different characters indicate significantly different groups within each clone ($p < 0.05$).

Relative gene expression of candidate genes within one clone

RNA was extracted immediately after hatching (up to one hour) of the neonate *D. pulex*, which had been exposed either to control water or *Chaoborus* kairomone containing water during embryogenesis. Relative gene expression was determined via qPCR. All gene expression levels in the kairomone treatments were normalized to their respective controls. For every clone, a separate one-way ANOVA was calculated. Equal variance tests were passed for all analysis. In case of *D. pulex* clone TCO, normality of the data was not given. Thus, a one-way ANOVA on ranks was calculated. Since each relative expression of kairomone induced genes was normalized to its respective control treatment, no cross gene comparisons were taken into account.

For clone *D. pulex* TCO (Fig. 2), a one-way ANOVA on ranks showed no statistically significant differences for the relative expressions of any of the candidate genes ($p = 0.095$, $H(13) = 20.017$). For *D. pulex* clone Gerstel (Fig. 3), a one-way ANOVA showed statistically significant differences of the relative expressions of the candidate genes ($p < 0.001$, $F_{1, 28} = 7.897$). Pairwise comparisons revealed a significant upregulation of the expression of the chitin deacetylase gene 1 (3.5-fold upregulation, Tukey`s HSD $p = 0.002$) and chitin deacetylase gene 2 (4-fold upregulation, Tukey`s HSD $p < 0.001$) due to the exposure to *Chaoborus* kairomone. The other candidate genes did not show any significant changes. For *D. pulex* clone Münster (Fig. 4), the one-way ANOVA showed significant differences of the relative expressions of the candidate genes ($p < 0.004$, $F_{1, 28} = 3.234$). However, the pairwise comparison of the control and kairomone treatments for the respective candidate genes failed to show any significant up- or downregulation.

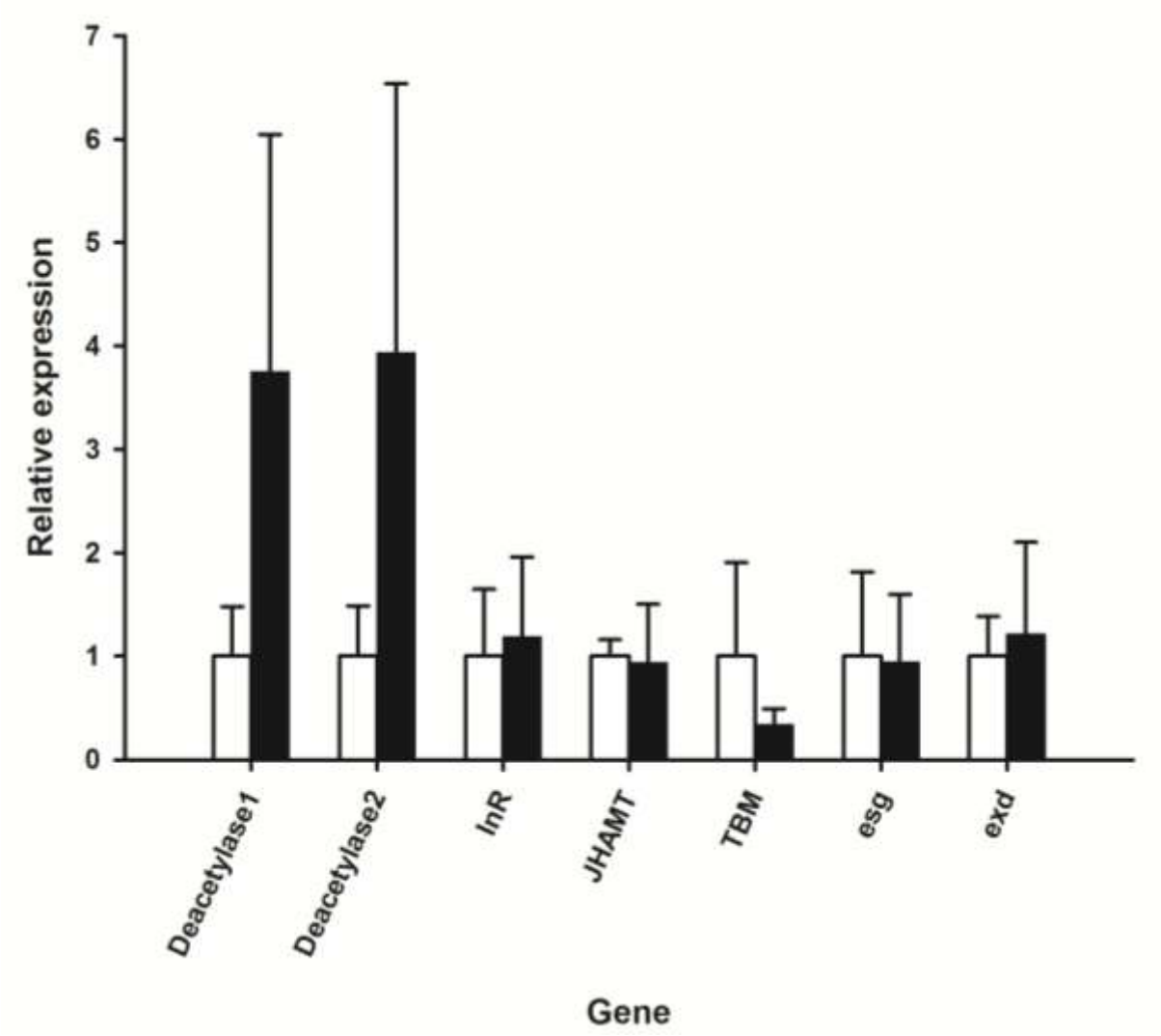

Figure 2: Mean (n = 3, + SD) relative expression of candidate genes in *D. pulex* clone TCO. Abbreviations are: *Chitin deacetylase* 1 and 2 (*deacetylase*), *insulin-like receptor* (*InR*), *juvenile hormone methyl transferase* (*JHAMT*), *tyramine beta-monooxygenase* (*TBM*), *escargot* (*esg*) and *extradenticle* (*exd*). Expression was determined immediately after hatching of the neonates that had been exposed to either control water (white bars) or water containing *Chaoborus* incubation water extract (black bars).

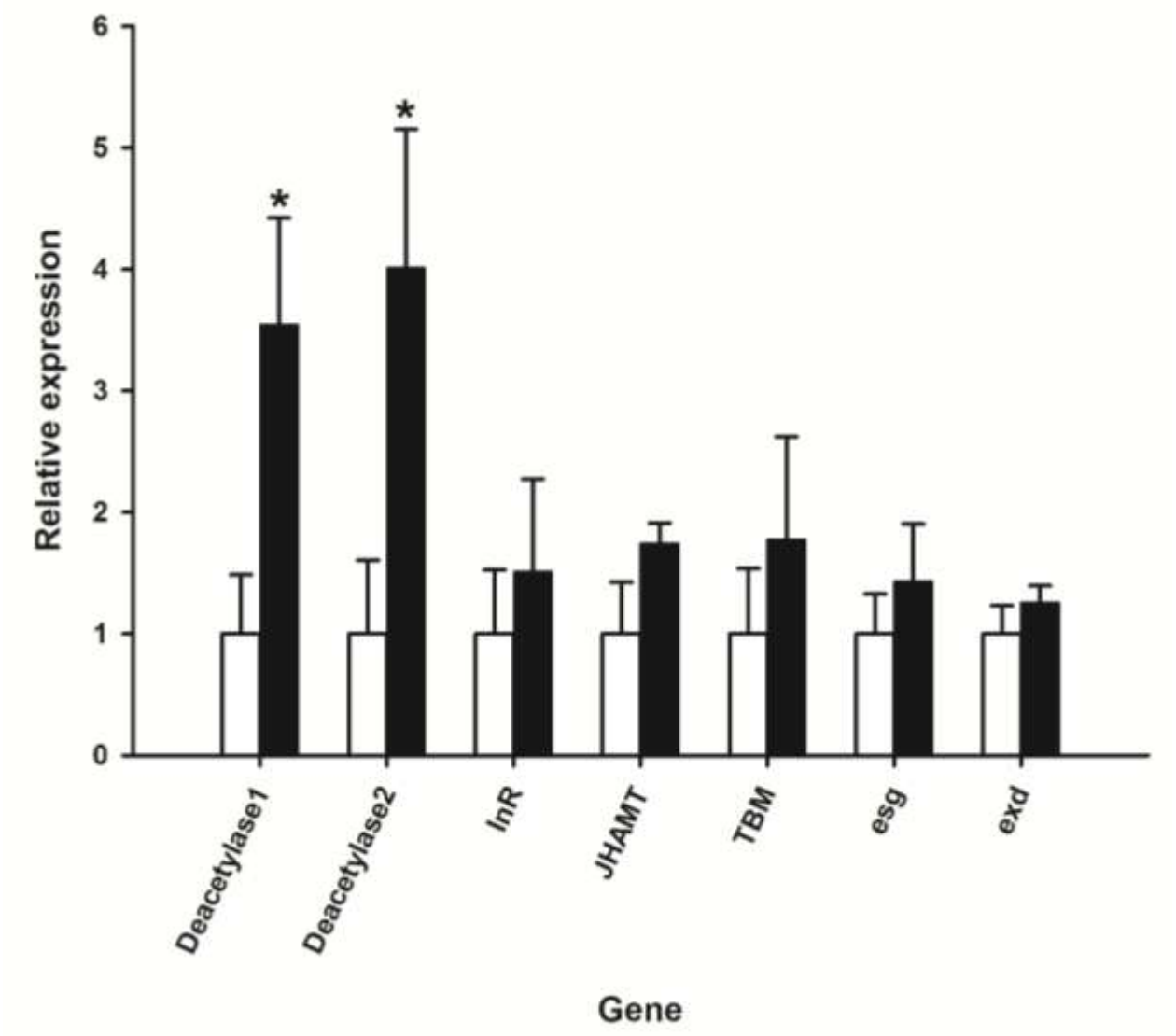

Figure 3: Mean (n = 3, + SD) relative expression of candidate genes in *D. pulex* clone Gerstel. For abbreviations see legend figure 2. Expression was determined immediately after hatching of the neonates that had been exposed to either control water (white bars) or water containing *Chaoborus* incubation water extract (black bars). Asterisks indicate a significant difference between control and kairomone treatments (t-test: $p < 0.05$).

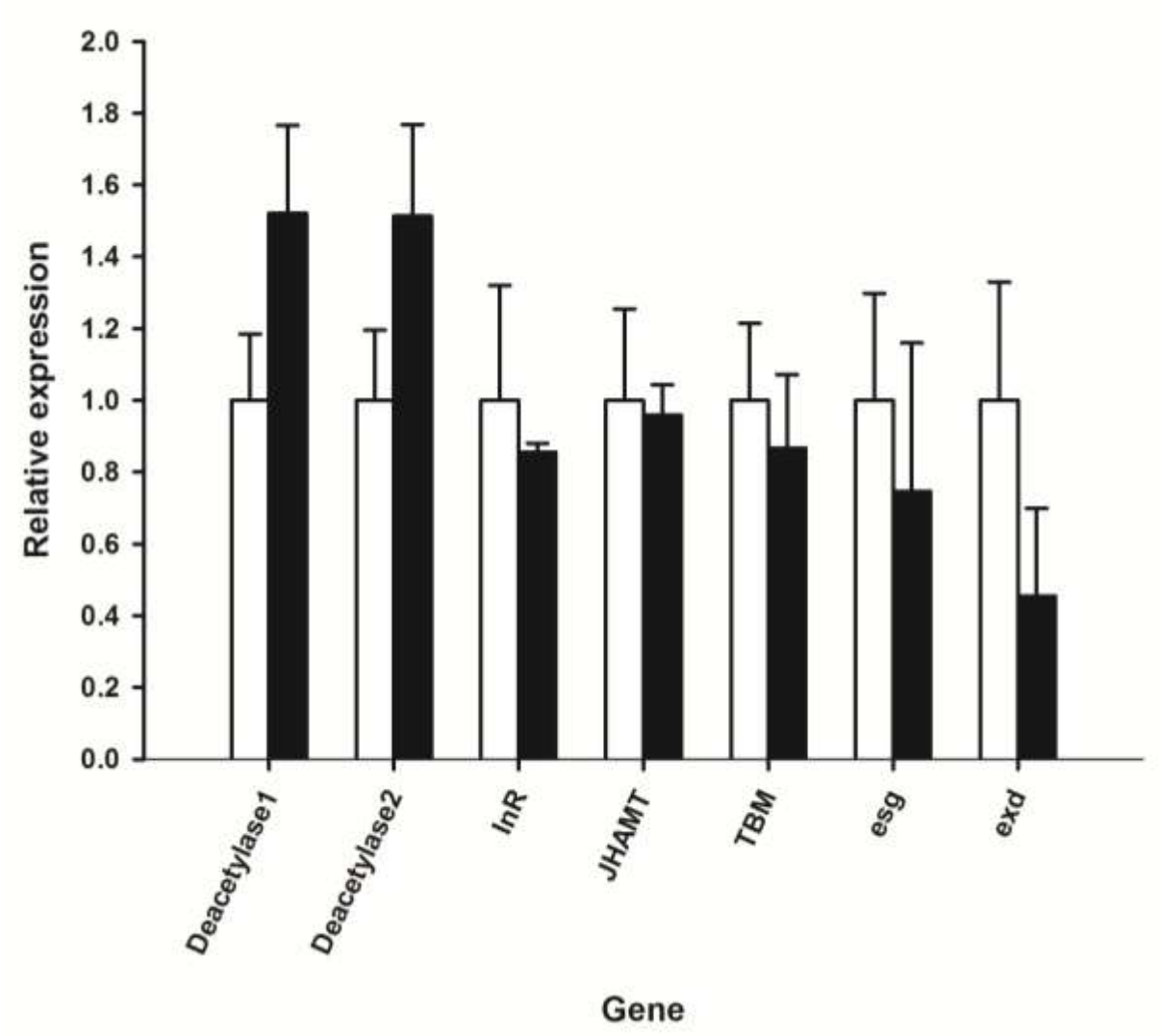

Figure 4: Mean (n = 3, + SD) relative expression of candidate genes in *D. pulex* clone Münster. For abbreviations see legend figure 2. Expression was determined immediately after hatching of the neonates that had been exposed to either control water (white bars) or water containing *Chaoborus* incubation water extract (black bars).

Cross clone comparison of deacetylase genes

Gene expression levels of the chitin deacetylase 1 (Fig. 5) were compared between all *D. pulex* clones with and without kairomone by means of a one-way ANOVA. All gene expression levels were normalized to the clone TCO control treatment. Normality test ($p = 0.641$) and equal variance test ($p = 0.745$) were passed. The ANOVA revealed significant differences between the treatments ($F_{5, 12} = 13.333$, $p < 0.001$). The kairomone treatment of clone Münster had the overall highest relative gene expression (8.4-fold increase compared to

the clone TCO control treatment, Tukey HSD: $p < 0.001$). Further, gene expression of the deacetylase 1 in the control treatment of clone Münster (5.5-fold increase) and the kairomone treatment of clone Gerstel (5.4-fold increase) were also significantly upregulated compared to the clone TCO control treatment (Tukey HSD: $p = 0.014$ for clone Münster control treatment and $p = 0.015$ for clone Gerstel kairomone treatment). On the other hand, the gene expression in the control treatment of clone Gerstel (Tukey HSD: $p = 0.367$) and in the kairomone treatment of clone TCO was not upregulated (Tukey HSD: $p = 0.193$).

Results for chitin deacetylase 2 were quite similar (Fig. 6). All gene expression levels were normalized to the TCO control treatment. Normality test ($p = 0.757$) and equal variance test ($p = 0.621$) were both passed. The ANOVA revealed significant differences between the treatments ($F_{5, 12} = 10.581$, $p < 0.001$). The overall pattern was identical with only slightly differing expression levels and p values compared to chitin deacetylase 1. The kairomone treatment of clone Münster showed the highest upregulation (8.1-fold, Tukey HSD: $p < 0.001$). The gene expression in the control treatment of clone Münster (5.3-fold, Tukey HSD: $p = 0.029$) and the kairomone treatment of clone Gerstel were also upregulated (5.2-fold, Tukey HSD: $p = 0.038$), whereas the control treatment of clone Gerstel (Tukey HSD: $p = 1.000$) and the kairomone treatment of clone TCO (Tukey HSD: $p = 0.203$) were not.

Relative gene expression as a function of neck-teeth induction

When the expression levels of the chitin deacetylase genes 1 and 2 were depicted as a function of the neonate neck-teeth induction values of the respective treatments across all *D. pulex* clones and treatments, a regression analysis revealed a significant linear correlation between both parameters for chitin deacetylase 1 (Fig. 7, $F_{1, 5} = 17.914$, $p = 0.013$, $r^2 = 0.82$) and chitin deacetylase 2 (Fig. 8, $F_{1, 5} = 16.534$, $p = 0.015$, $r^2 = 0.81$). A normal distribution of the data was given (Shapiro-Wilk normality test, for chitin deacetylase 1 $p = 0.505$ and for chitin deacetylase 2: $p = 0.481$).

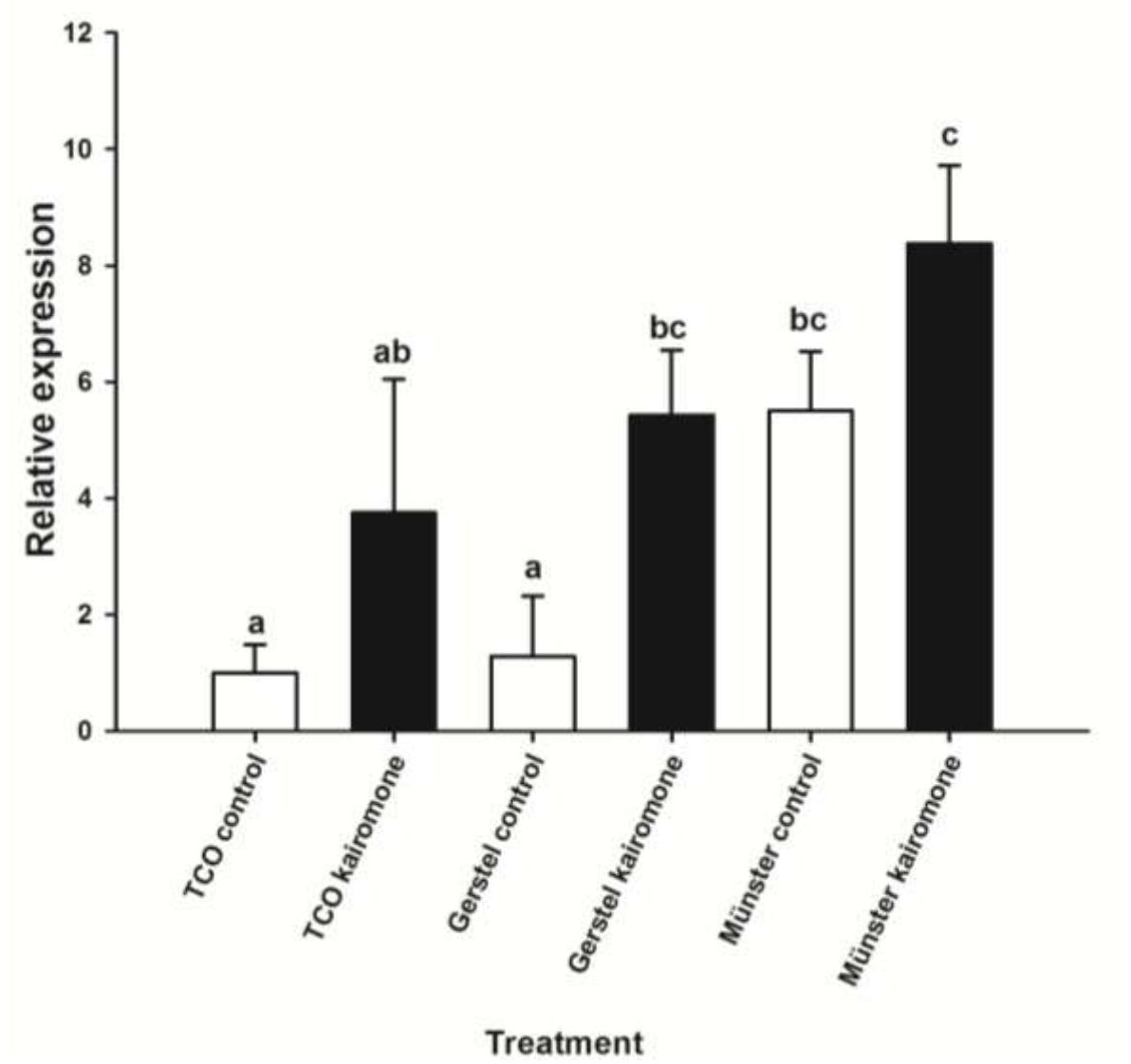

Figure 5: Mean (n = 3, + SD) relative expression of *chitin deacetylase 1* in *D. pulex* in the three clones TCO, Gerstel and Münster normalized to TCO control. Expression was determined immediately after hatching of the neonates that had been exposed to either control water (white bars) or water containing *Chaoborus* incubation water extract (black bars). Different letters indicate significantly different groups (Tukey`s HSD: $p < 0.05$).

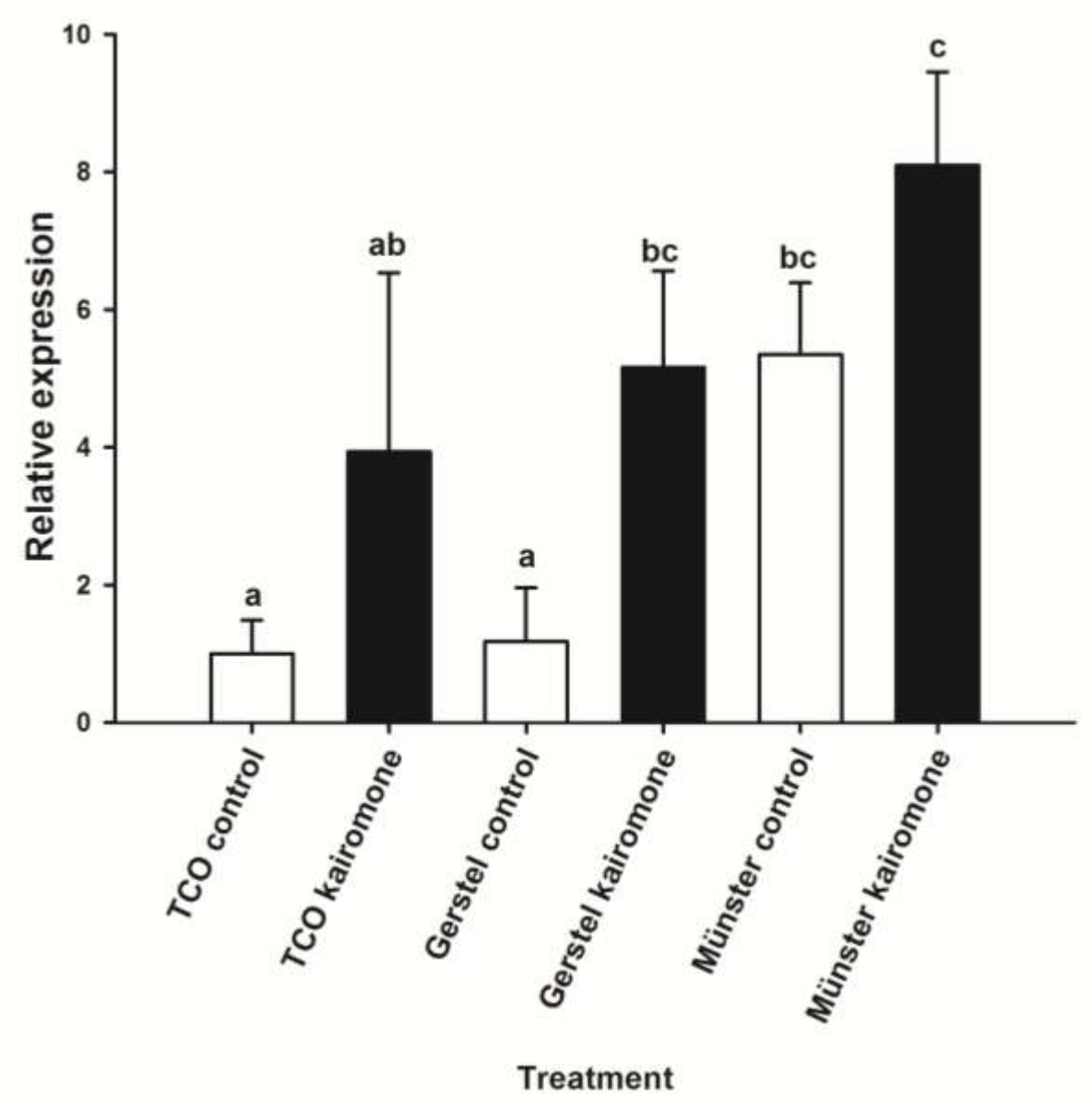

Figure 6: Mean (n = 3, + SD) relative expression of *chitin deacetylase 2* in *D. pulex* in all three clones (TCO, Gerstel and Münster) normalized to TCO control. Expression was determined immediately after hatching of the neonates that had been exposed to either control water (white bars) or water containing *Chaoborus* incubation water extract (black bars). Different letters indicate significantly different groups (Tukey`s HSD: $p < 0.05$).

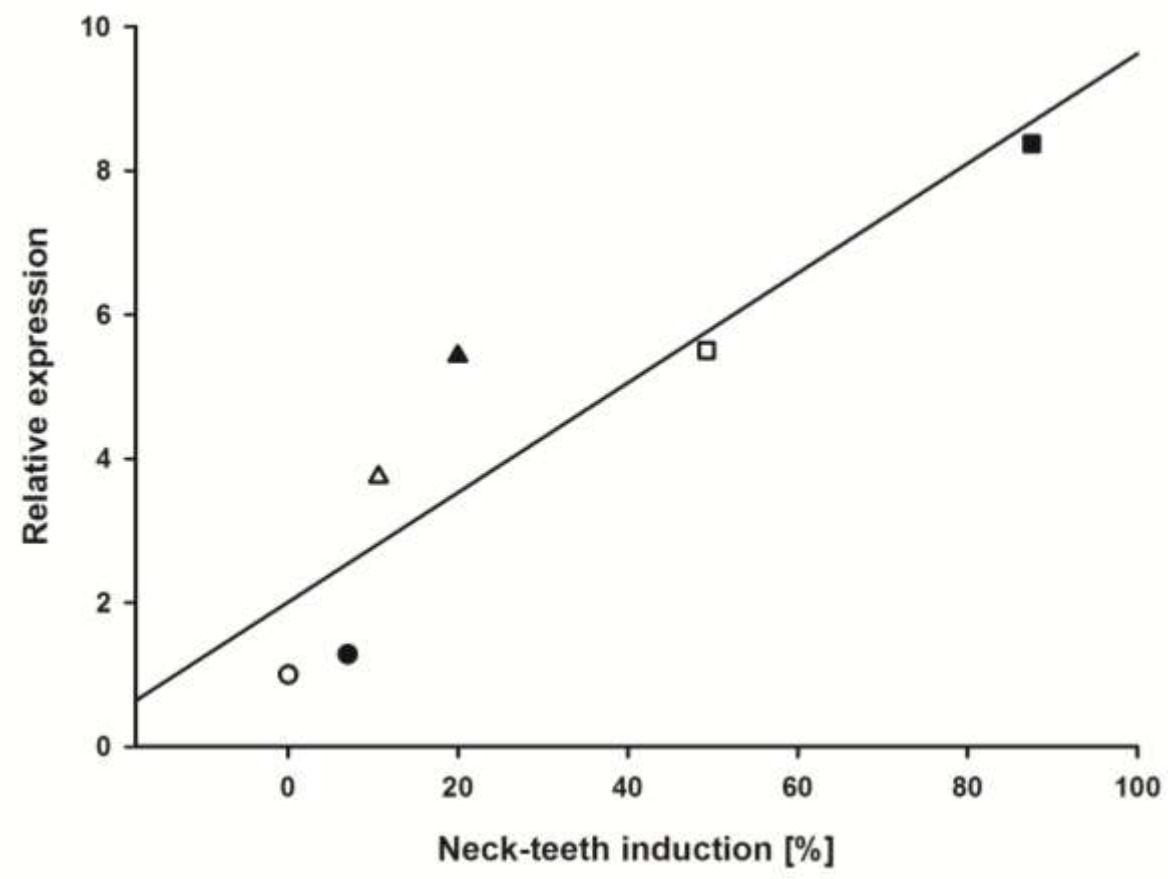

Figure 7: Relative gene expression of *chitin deacetylase 1* as a function of neck-teeth induction after hatching for neonates of *D. pulex* clone TCO (circles), clone Gerstel (triangles) and clone Münster (squares) both with (black symbols) or without kairomone (white symbols). r^2 values for the regressions line is 0.817.

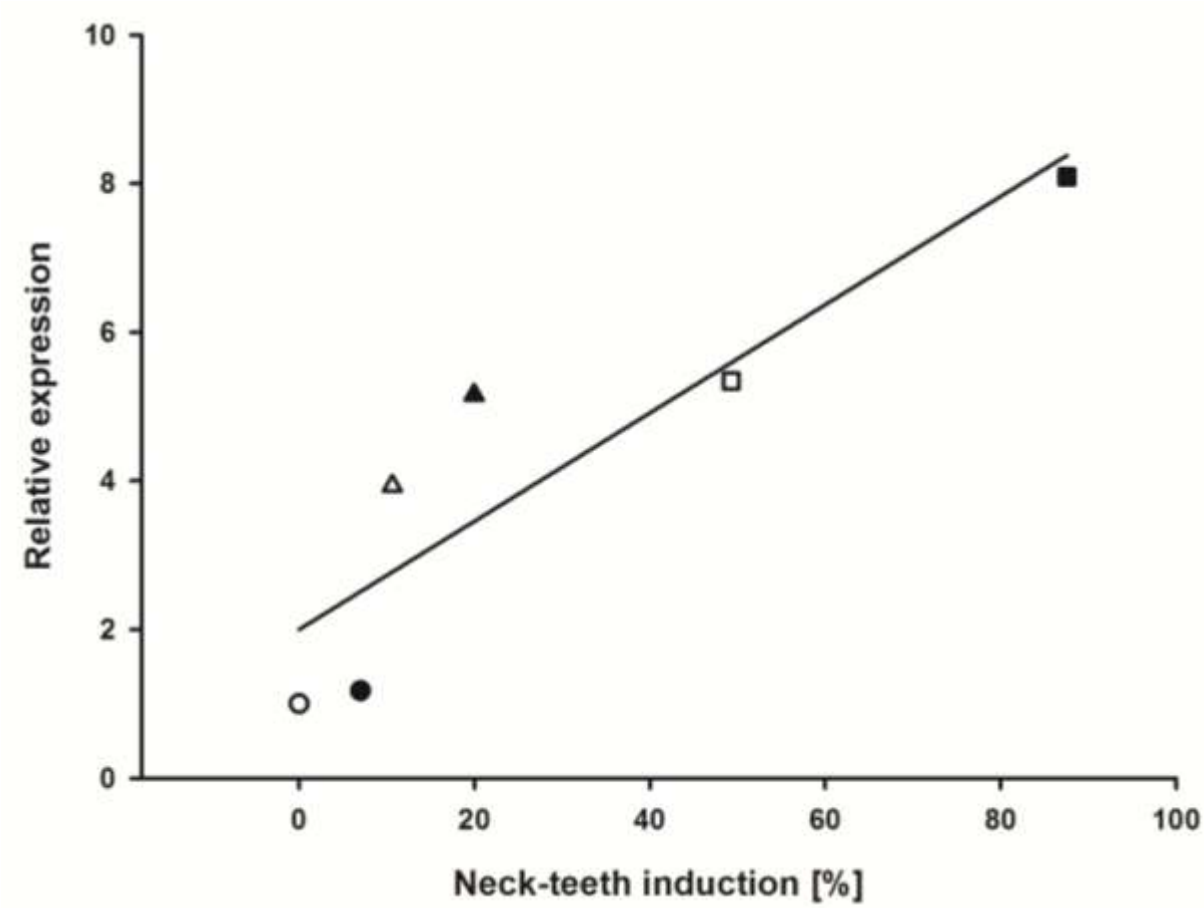

Figure 8: Relative gene expression of *chitin deacetylase 2* as a function of neck-teeth induction after hatching for neonates of *D. pulex* clone TCO (circles), clone Gerstel (triangles) and clone Münster (squares) both with (black symbols) or without kairomone (white symbols). r^2 values for the regressions line is 0.805.

Discussion

The presence of a predator, indicated by a chemical cue, imposes stress on potential prey organisms. In *Daphnia*, this may result in an increased alertness of the prey (Brewer *et al.* 1999), a change in behavior (Dodson 1988), changes of life history (Sakwinska & Dawidowicz 2005) or an altered morphology (Krueger & Dodson 1981). However, these inducible defences are not exclusively expressed, but have been shown to co-occur in many different combinations (Boersma *et al.* 1998). Therefore, the attribution of an upregulation of a candidate gene to a single inducible defence poses a challenging task, when several defences are expressed at the same time. By introducing the novel method of a gradient of morphologically differently responsive *D. pulex* clones and by correlating candidate gene expression profiles of several *D. pulex* clones with their respective strength of neck-teeth induction, new insights concerning the putative involvement of candidate genes could be obtained.

The three *D. pulex* clones used in this study differed in their neck-teeth expression significantly between kairomone and control treatments. Further, all kairomone treatments differed from one another as did all control treatments, providing a clonal gradient of neck-teeth development suited for the envisaged endeavour.

In none of the three investigated *D. pulex* clones a significant upregulation could be shown for neither of the genes proposed by (Miyakawa *et al.* 2010). A possible reason for this might be the fact, that (Miyakawa *et al.* 2010) sampled animals within the whole first 24 hours after hatching, whereas this study applied a much narrower time window of one hour only after hatching. Neck-teeth development is assumed to take place during this developmental step (Parejko 1992), and accordingly we assumed, that the expression of neck-teeth related genes is highest during the late phase of embryogenesis, when the first juvenile instar is nearly completely developed. This narrow time window chosen here is in accordance with the finding that fish-borne kairomones have been shown to induce upregulation of a specific set

of genes in *Daphnia* in a time frame of two hours only (Effertz & von Elert 2014). This finding suggests that the response in gene expression in *Daphnia* to kairomones released from larvae of *Chaoborus* is confined to a short time window and thus the affected genes may differ for different time windows, which might explain the discrepancies between Miyakawa et al. (2010) and this study. As the juvenile hormone pathway has been shown to be involved in the regulation of moulting (Chang *et al.* 1993), the reported upregulation of juvenile hormone / insulin signalling pathway genes during the first 24 hours after hatching by Miyakawa et al. (2010) might have been likewise caused by a predator induced shift in life history and may thus not necessarily be involved in neck-teeth induction.

There is a vast diversity of how strongly, or if at all, *D. pulex* clones respond to the presence of *Chaoborus* kairomone as they have been shown to vary widely with respect to the strength of neck-teeth induction (Lüning 1995). Therefore, neonates used for gene expression analysis in this study were examined concerning their strength of neck-teeth induction directly prior to RNA extraction. The *D. pulex* clone Münster has been shown to have a very high neck-teeth induction even in the absence of kairomones. This might lead to highly expressed neck-teeth genes in general with no, or only a slight, upregulation in the presence of kairomone. The broad candidate gene approach with only a single *D. pulex* clone performed by (Miyakawa *et al.* 2010) might therefore miss genes actually involved in neck-teeth induction, and mistake genes which are in fact unrelated to the regulation of neck-teeth induction falsely for being involved. As Miyakawa et al. (2010) did not provide information on the strength of neck-teeth induction of the *D. pulex* clone used in their study, or the difference of neck-teeth induction between uninduced and induced morph, the reported differences in relative gene expressions cannot be unambiguously be attributed to the regulation of neck teeth, as they might be involved in a general stress response or be part of another induced defence on the behavioural or life-history level.

Using a candidate gene approach, (Miyakawa *et al.* 2010) proposed the involvement of the juvenile hormone and insulin signalling pathway in the regulation of neck-teeth induction. In addition, juvenile hormone pathway genes (juvenile hormone acid methyltransferase and methoprene-tolerant) and genes of the insulin signalling pathway (insulin-like receptor and insulin receptor substrate-1) were found to be upregulated in neonates of *D. pulex.* An involvement of the juvenile hormone pathway was further supported by (Miyakawa *et al.* 2013), who reported an increase of kairomone dependent neck-teeth induction in the presence of juvenile hormone. The involvement of the juvenile hormone pathway in neck-teeth induction was also addressed by (Dennis *et al.* 2014), who proposed a small network of genes. Using a single clone of *D. pulex*, Dennis et al. 2014 showed a dose-dependent upregulation of the genes *EcRb*, *HR3* and *HB2* in response to three different concentrations of *Chaoborus* kairomone; this represents a pattern of expression, which is in good agreement with the juvenoid expression profile. As no statistical data is presented in their study, the dose dependent upregulation of *HB2* remains questionable, as the intermediate kairomone concentration seems to lead to a lower gene expression than the low kairomone concentration. Nevertheless, *EcRb* and *HR3* have been shown to be essential for moulting in *Tribolium* (Tan & Palli 2008) with *EcRb* and *HR3* being nuclear receptors and *HB2* being a haemoglobin gene. Unfortunately, (Dennis *et al.* 2014), too, did not provide data on the strength of neck-teeth induction in the animals used in their assay, which all leaves the two incidents of effectively induced neck-teeth and the dose-dependent increase in relative gene expression unconnected. As no dose dependent increase of neck-teeth induction is reported, the dose dependent increase in gene expression must not be unambiguously attributed to be correlated to a regulation of the morphological defence.

The indication of an involvement of the juvenoid pathway as well as the insulin signalling pathway in the regulation of neck-teeth induction is not supported by data presented in this study. This might possibly be due to the fact that (Dennis *et al.* 2014) used frozen *Chaoborus*

larvae which were boiled for the preparation of their kairomone containing extract, whereas in this study *Chaoborus* incubation water was extracted. The boiling of larvae probably leads not only to the release of kairomone but also to the liberation of a vast number of other substances that are naturally contained in the larval tissue such as juvenile hormones. As these molecules belong to a group of non-polar substances, the subsequent C_{18} solid phase extraction performed by (Dennis *et al.* 2014) would lead not only to an enrichment of the *Chaoborus* kairomone but also of juvenile hormones, as shown by (Grossniklaus-Bürgin & Lanzrein 1990). Thus it seems reasonable to assume that the addition of larval extract in Dennis et al. (2014) might have induced an upregulation of genes of the juvenile hormone pathway in *D. pulex* due to the unintentional presence of juvenile hormones in the larval extract.

In conclusion the evidences presented in (Miyakawa *et al.* 2010) and (Dennis *et al.* 2014) do not necessarily / unambiguously provide proof for an involvement of the juvenile hormone pathway or the insulin signalling pathway in predator induced morphological changes in *D. pulex*. Here, although five genes either directly involved in the juvenoid/insulin signalling pathway or genes supposedly downstream of them (Miyakawa *et al.* 2010) were investigated, no evidence for a role of both hormone pathways in the induction of morphological changes by larvae of *Chaoborus* was obtained.

In addition to the candidate genes that were used according to (Miyakawa *et al.* 2010), we also investigated effects of *Chaoborus* kairomone on the expression of two chitin deacetylases. Expression of these two genes was found to be upregulated exclusively in D. pulex clone Gerstel in response to *Chaoborus* kairomone, which has an intermediate strength of neck-teeth induction among the three *D. pulex* clones used in this study. In *D. pulex* clone TCO, no neck-teeth were detectable in the control treatment, and only a very low induction was found in the kairomone treatment. In *D. pulex* clone Münster, on the other hand, even neonates from the control treatment showed a strong neck-teeth induction, which was significantly higher than kairomone treatments from *D. pulex* clone TCO and *D. pulex*

Gerstel. The kairomone treatment of *D. pulex* clone Münster had the strongest neck-teeth induction of all treatments. Surprisingly, this strong morphologic defence seemed not to be reflected on the gene level. However, when comparing chitin deacetylase gene expressions in all treatments and in all three *D. pulex* clones, the normalized gene expression revealed a positive correlation between strength of neck-teeth induction and gene expression along the clonal gradient. This was similar for both chitin deacetylase genes with only minor differences. Generally, this pattern of expression of chitin deacetylase genes fits the picture proposed by (Beckerman *et al.* 2013), who argued that the arthropod response to predator kairomones is based on the regulation of the chitin synthesis and degradation. The chitin metabolism is centred on the temporal and spatial modifications of the carapace, which is known to be chitin rich. Chitin deacetylases are chitin modifying enzymes, known to catalyze the N-deacetylation of chitin to influence the protein binding affinity of chitin filaments. In *Tribolium castaneum*, chitin deacetylases of type 1 and 2 are mainly expressed in the exoskeletal epidermis (Arakane *et al.* 2009), which would be in accordance with an involvement of chitin deacetylases in the expression of morphological defences.

The results of this study promote the importance of chitin related genes for the development of morphological defences in *Daphnia*. Although chitin deacetylases are probably not the basal gene responsible for the regulation of neck-teeth induction, the more than eight-fold upregulation in the induced clone *D. pulex* Münster compared to the uninduced *D. pulex* clone TCO indicates the importance of these deacetylases. Further research is needed to completely understand the molecular mechanics of morphological defences in *Daphnia*, and this study offers a useful approach by introducing the use of a clonal neck-teeth gradient to differentiate gene expression of neck-teeth specific genes from genes that are part of other defence related pathways or are upregulated due to general predator imposed stress.

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Chapter 3

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Prey-induced vertical migration in *Chaoborus* larvae under different predator and light regimes

Abstract

For freshwater prey like *Daphnia* and larvae of *Chaoborus* it is well documented that they adjust their residence depth in the water column in response to predator kairomones in order to decrease encounter probability with the respective predator. Despite the importance of infochemicals in predator-prey interactions, it has not been tested if predators adjust their residence depth in response to infochemicals released by prey. Here we use an indoor system with a stratified water column and show that the predatory phantom midge *Chaoborus* prefers strata with *Daphnia* incubation water over strata with control water. Further, the chemically mediated effect of a top predator (fish) on this system was shown to be light dependent with *Chaoborus* avoiding prey conditioned water when it also contained fish kairomone in brighter surface water, but not in deeper and thus darker water layers. The foraging kairomone released by *Daphnia* can be extracted from incubation water via C_{18} – solid phase extraction. These results add another dimension to the steering role of infochemicals in predator-prey interactions in zooplankton.

Introduction

Predation is one of the major driving forces that shape natural communities (Zaret 1980), and therefore understanding of the mechanisms of predator-prey interactions is essential to fully comprehend whole ecosystems. In standing freshwaters, the keystone crustacean *Daphnia* is a major prey for higher trophic levels, and frequently the larvae of the phantom midge *Chaoborus* are the major invertebrate predator of daphnids. However, the interaction of larvae of *Chaoborus* with *Daphnia* has largely been studied with respect to the high phenotypical plasticity of *Daphnia*. Daphnids have been shown to react to infochemicals released by their predators, so called kairomones, with various defence strategies. Oram and Spitze (Oram and Spitze, 2013) demonstrated that neonates of *D. pulex* position themselves higher in the water column when exposed to *Chaoborus* kairomone compared to control treatments without kairomone. Besides this behavioural defence, exposure to *Chaoborus* kairomone induces changes in *Daphnia*'s body morphology by enlargement of heads (Tollrian, 1990) or tail spines (Dzialowski et al., 2003) or by development of new structures like neck-teeth that reduce mortality due to *Chaoborus* predation (Havel and Dodson 1984). Thus *Daphnia* seem to be provided with a plethora of mechanisms to defend against *Chaoborus* larvae and reduce the larvae's foraging success. Kotov and Taylor (Kotov and Taylor, 2011) investigated fossils of ephippia and Chaoboridae associated with the Jurassic-Cretaceous boundary, suggesting a coevolution of both groups since 145 million years. Thus, it seems reasonable, that *Chaoborus* larvae have evolved means of increasing their efficiency when preying upon *Daphnia*.

One means of achieving this might be the utilisation of chemical cues released by *Daphnia* for increasing the encounter probability of *Chaoborus* larvae with daphnids. Since the vertical position of *Daphnia* in the water column changes seasonally and diurnally, it is reasonable to assume that larvae of *Chaoborus* are able to sense the vertical presence of *Daphnia* and to adjust their own vertical position in the water column accordingly. Chemical compounds,

which are released by the prey and that mediate such a food-finding response in the predator are termed foraging kairomones. In aquatic systems Fink et al. (Fink et al., 2006) reported an example of foraging kairomones in the aquatic realm: They showed that the pulmonate snail *Radix ovata* is attracted to volatile organic compounds released from disintegrated cells of the benthic, mat-forming green algae *Ulothrix fimbriata*. This system is even more complex as shown by Moelzner and Fink (Moelzner and Fink, 2014), who demonstrated that snails are even able to differentiate between food of high and low quality by recognizing quality specific bouquets of volatile organic compounds. With respect to larvae of *Chaoborus* it has been shown that fourth instar larvae of both *C. americanus* and *C. punctipennis* increased their movement frequency when being exposed to prey conditioned water (Berendonk and Obrien, 1996). However, the prey consisted of a mix of daphnids and copepods, making it impossible to attribute the observed effect to either prey alone. Furthermore, *Chaoborus* larvae are a sit-and-wait predator, and it remains unclear if prey conditioned water would affect the vertical position of *Chaoborus* in the water column. Here we investigate, if *Daphnia* release chemical cues that affect the vertical position of *Chaoborus* larvae in a stratified water column (Table 1).

However, larvae of *Chaoborus* are not the top predator in aquatic foodwebs. Vertebrates like the Eurasian perch (*Perca fluviatilis*) have been shown to prey heavily on *Chaoborus* larvae (Regmi et al., 2013). In controlled lab experiments Dawidowicz et al. (Dawidowicz et al., 1990) showed that water, which had previously contained fish (*Gasterosteus aculeatus* L.), affected the day and night time vertical position of *Chaoborus flavicans* larvae such that the larvae were moving to deeper strata in the water column, where detectability for optically hunting fish would be reduced. And although much is known about fish induced DVM in *Chaoborus*, the infochemically mediated effects of the simultaneous presence of a predator and a prey on a species remain poorly understood. As, in many cases, larvae of *Chaoborus* coexist with predators (planktivorous fish) and *Daphnia* as prey, we here further investigate

the combined effects of kairomones from a predator and from a prey on the vertical position of *Chaoborus* larvae in the water column.

Therefore, experiments were designed to test whether the application of *Daphnia* incubation water at the top or bottom end of an experimental tube will affect the daytime vertical position of *Chaoborus* larvae. Subsequently, with the insights gained from these experiments, additional trials were conducted, which incorporated not only *Daphnia* incubation water but also fish kairomones in order to simulate more ecologically relevant and thus natural conditions with both a predator and a prey present.

Finally, it was tested if the *Daphnia* borne kairomone could be enriched by using C_{18} – based solid phase extraction. On one hand, C_{18} extraction has been proven to be a useful tool for the enrichment of the kairomones from incubation water of both *Chaoborus* (Tollrian and von Elert, 1994) and fish (von Elert and Pohnert, 2000)(von Elert and Loose, 1996), which have been shown to induce various defences in *Daphnia*. This opportunity to enrich kairomone from incubation water and to store strongly concentrated kairomone allows for future research with standardized kairomone concentrations. However, very little is known about the chemical features of any of the existing aquatic kairomones (von Elert, 2012). However, to fully comprehend the complex predator-prey systems, more detailed information on the identity of the substances is needed. This study helps to initiate the research on the chemical features of the *Daphnia* kairomone.

Table 2: Results of the one-way analyses of variances on the mean vertical positions of the *Chaoborus* larvae. Asterisks indicate a significant difference with $p < 0.05$

Experiment	SS	df	F	P	
Daphnia incubation water at top and bottom					
Treatment	4973.60	1	27.3939	< 0.001	*
Error	3631.18	20			
Fish incubation water extract with controls					
Treatment	641.74	2	21.109	< 0.001	*
Error	228.01	15			
Daphnia incubation water with fish incubation water extract					
Treatment	6364.0	3	13.8570	< 0.001	*
Error	5511.1	36			
Daphnia incubation water SPE permeate					
"Var1"	5998.54	3	26.996	< 0.001	*
Error	2666.39	36			
Daphnia incubation water SPE extract					
"Var1"	7195.4	3	22.3814	< 0.001	*
Error	3750.7	35			

Material and methods

Animals

Fourth instar larvae of *Chaoborus obscuripes* were obtained from an internet pet shop (Interquaristik.de). Prior to the experiments, the animals were transferred to aged and aerated tap water and starved for at least one day to increase foraging motivation. *Daphnia magna* clone B originated from the Großer Binnensee in Germany, N 54.324828, E 10.629541 (Lampert and Rothhaupt, 1991). For the preparation of *Daphnia* incubation water, between 300 and 450 adult *D. magna* per litre were kept in 10 L of aged and aerated tap water and fed with 2 mg C/L *Chlamydomonas klinobasis*. After 24 hours, the *Daphnia* were sieved off, and the incubation water was filtered with glass fibre filters (MN 85/220, pore size approximately 0.4 µm, 47 mm diameter, Macherey & Nagel, Düren, Germany). Control water with only 2 mg C/L of *Chlamydomonas klinobasis* but without *Daphnia* was treated identically.

Daphnia Food

Daphnia were fed the green alga *Chlamydomonas klinobasis*, strain 56, from the culture collection of the work group. *C. klinobasis* was grown in 5 L semi-continuous batch cultures (20°C; illumination: 120 mmol photons per second and square meter) by replacing every other day 20% of the culture with fresh, sterile Cyano medium (Elert and Juttner, 1997), to which 1 mL of a vitamin stock solution (modified according to (Guillard et al., 1975)) had been added per litre of medium. Subsequently the suspension was filtered through a 30 µm gauze to remove particles that cannot be filtered by *Daphnia*.

Extracts of fish incubation water

Three *Perca fluviatilis* (body size: 10–12 cm) were pre-conditioned for 24 h without food and then kept for another 24 h in 8 L of aged tap water at 18°C without feeding. The incubation water was filtered through glass fibre filter (Whatman, MN 85/220, pore size approximately 0.4 µm, 47 mm diameter, Macherey & Nagel, Düren, Germany). For bulk enrichment of the kairomones from the incubation water as performed according to (von Elert and Stibor, 2006):

a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 mL, end-capped, Varian Mega Bond Elut, Agilent Technologies) was pre-conditioned with 50 mL methanol and 50 mL ultrapure water prior to adding the sample. Methanol was added to the incubation water to obtain a 1% concentration, and a volume of 2 L was passed through the cartridge. The loaded cartridge was then washed with 50 mL of ultrapure water and eluted with 50 mL of methanol. The eluates originating from 10 L of fish incubation water were pooled and evaporated to dryness using a rotary evaporator and then redissolved in 1 mL of absolute ethanol. Water without fish was used for the production of a control extract. The same standardized extracts of control water and fish incubation water were used for all experiments. For all experiments, 100 µL of extract of control water or of fish incubation water were dissolved per 1 L of aged tap water used in the experimental tubes.

Bioassay in plankton organ

The behavioural assays used a setup developed by Dawidowicz and Loose (Dawidowicz and Loose, 1992) and modified such that it was run in batch instead of flow-through mode. The experiments were carried out in Perspex tubes (1 m height, 200 mL volume), which were placed in a water bath with a defined temperature stratification (Fig. 1) mimicking a stratified lake. The tubes were illuminated from the top. To secure stratification in the experimental tubes in order to provide foraging and predator kairomones either only in the upper or in the lower stratum, the tubes were filled first with 50 mL precooled water of 4°C. Subsequently, 150 mL of water with room temperature were carefully added.

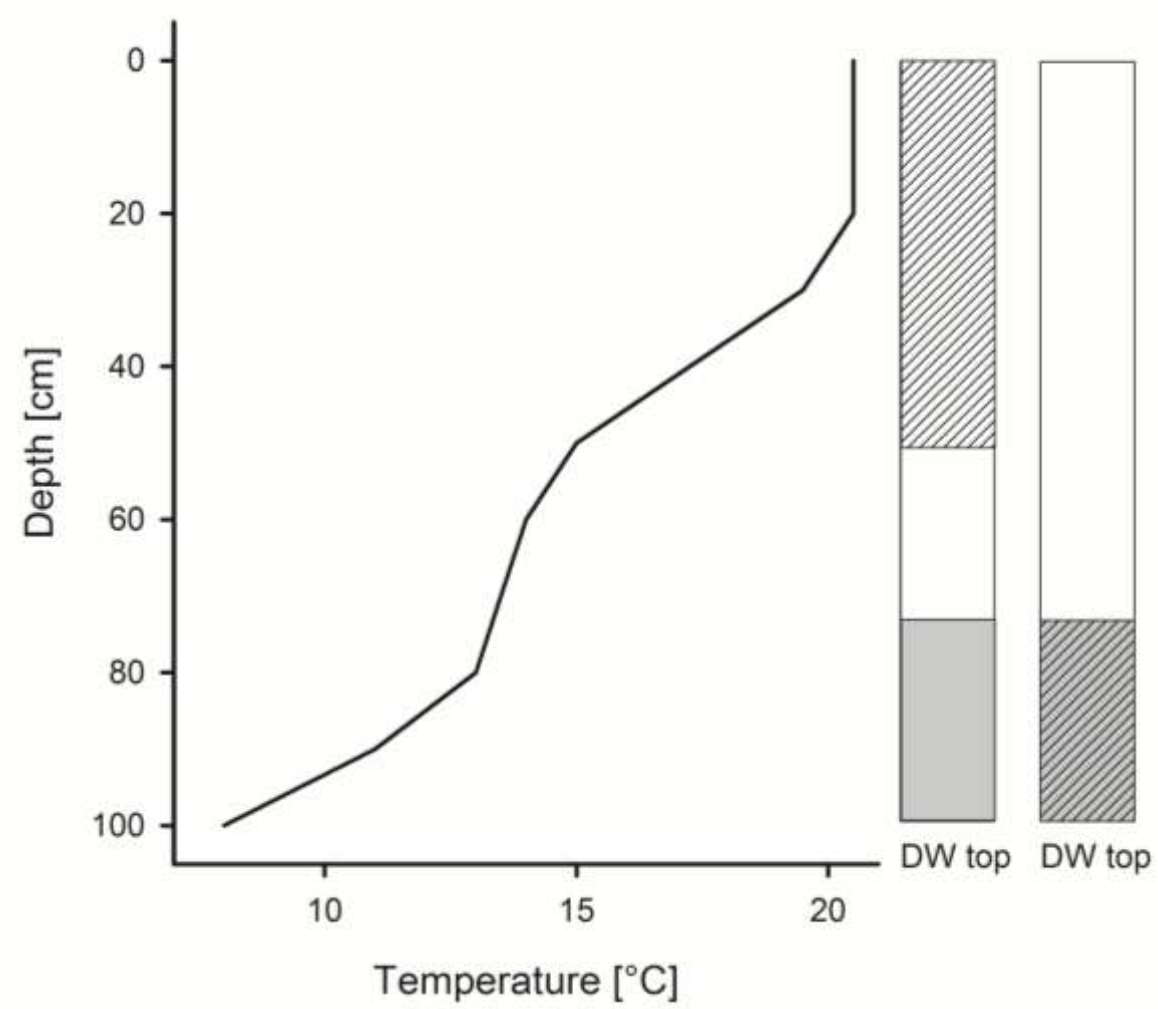

Figure 7: Temperature conditions in the plankton organ. The two boxes on the right illustrate how differently tempered water was applied to the experimental tubes to ensure a stratification of incubation water and control water. White corresponds to water at room temperature and grey to precooled water at 4°C. Shaded areas mark the appliance of *Daphnia* incubation water; areas without shades are control water.

For all bioassays, the vertical positions of *C. obscuripes* larvae were recorded five hours after the introduction of the *Chaoborus* larvae. All experiments were carried out under daytime light conditions with 6 to 8 *Chaoborus* larvae. This irregular number resulted from mortality which occurred probably due to the unfavourable conditions during the transportation and took place randomly in all treatments. Mean values of vertical positions of all larvae in one tube were calculated and treated as one biological replicate.

To test for a reaction of *Chaoborus* larvae to kairomones released by fish, larvae were placed in each experimental tube that contained either aged tap water (negative control), control water extract or fish incubation water extract. All treatments were replicated six fold.

In order to investigate the effect of *Daphnia* incubation water on the vertical position of *Chaoborus* larvae, 50 mL precooled incubation water were transferred into to lower quarter of the experimental tubes. The tubes were then carefully filled with control water at room temperature. This treatment is from now on referred to as "DW bottom". Another set of tubes were filled with precooled control water in the lower quarter of the experimental tubes, and then a layer of 50 mL control water of room temperature was gently poured on top. The top half of the experimental tubes was then filled with 100 ml *Daphnia* incubation water of room temperature. This treatment is from now on referred to as "DW top". All treatments were replicated eleven fold.

As the predator (fish) and the prey (*Daphnia*) are often present simultaneously under natural conditions, a combined experimental setup was designed, in which *Chaoborus* larvae were exposed to kairomones from fish and *Daphnia* at the same time. Therefore *Chaoborus* larvae were exposed to prey conditioned water either exclusively (DW top, DW bottom) or to prey conditioned water spiked with fish incubation water extract. All treatments were replicated tenfold

In order to test if the biological activity of *Daphnia* incubation water can be enriched by solid phase extraction, *Daphnia* incubation water was extracted via by C_{18} solid phase extraction as described above. Control water was treated identically. From both control and *Daphnia* incubation water the C_{18} permeates were collected and evaporated to dryness to remove the methanol that had been added prior to the extraction. The dried residue, which originated from 1 litre of incubation water, was redissolved in 500 mL ultrapure water only to balance potential losses during the processing. Two separate bioassays were carried out; one with *Chaoborus* larvae subjected to the C_{18} permeates of control water and *Daphnia* incubation

water, one with larvae subjected to the C_{18} extracts either from control or *Daphnia* incubation water. Each bioassay was performed with control water as negative control and *Daphnia* incubation water as positive control. All tested controls, permeates and extracts were applied as precooled volumes of 50 mL to the lower strata of the experimental tubes. The tubes were then filled up with control water, and the experiments were replicated tenfold.

Statistics

All data were checked for homoscedasticity (Levene`s Test). If this was the case, a t-test (in case of two treatments) or an analyses of variance (one-way ANOVA, in case of more than two treatments) and post-hoc tests (Tukey) were conducted. All statistical calculations were carried out the program "STATISTICA" (StarSoft Inc., V 6.0). The significance level had been set to $p < 0.05$ for all conducted tests.

Results

When incubation water of a planktivorous fish (*Perca fluviatilis*) was extracted by a lipophilic solid phase, the resultant extract induced a deeper daytime residence (55 cm) of *Chaoborus* larvae in the stratified water column than a control extract (41 cm) and a negative control (42 cm) did (Fig. 2, one-way ANOVA: $p < 0.0005$; $F_{2, 15} = 21.12$ followed by Tukey's HSD). The vertical position of the larvae did not differ between the two controls (Tukey's HSD: $p = 0.71$).

Chaoborus larvae in the control treatments experienced a mean temperature of approximately 16.8 °C, whereas those exposed to extract of fish incubation water resided at a mean temperature of 14.6 °C.

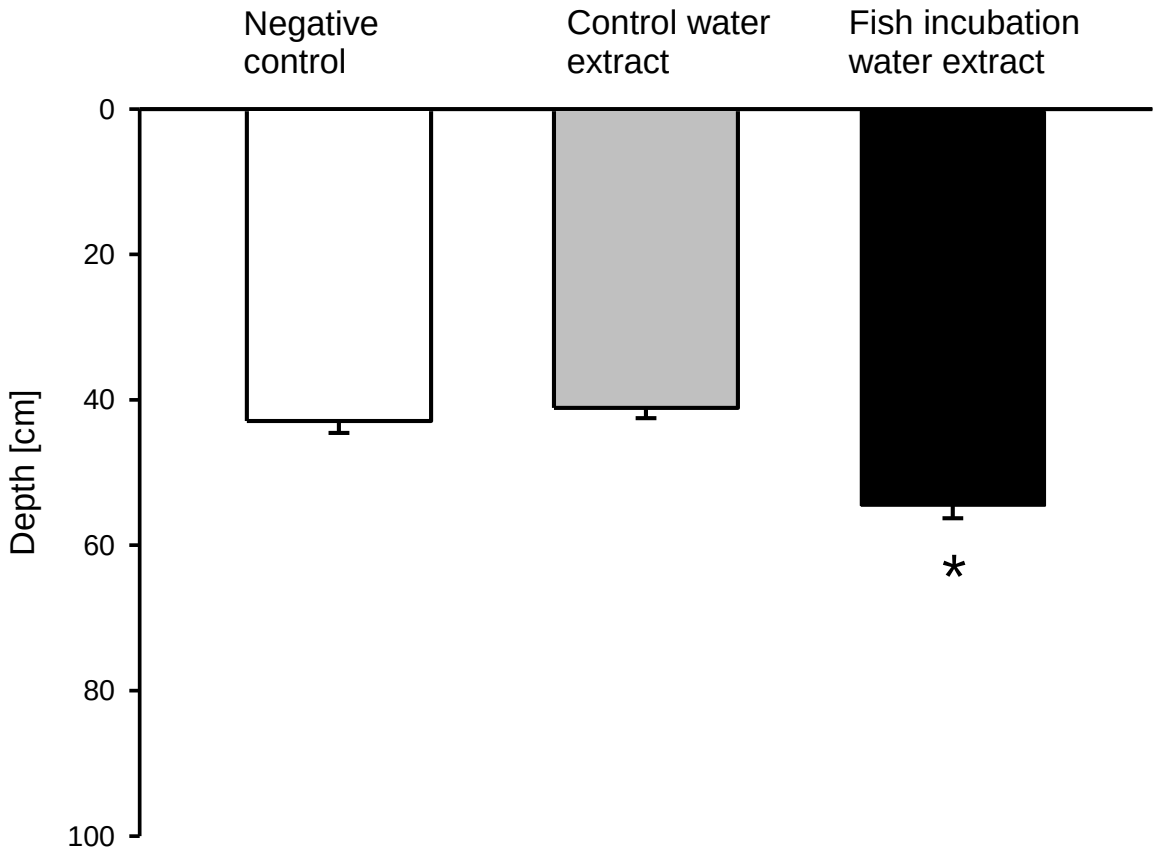

Figure 8: Mean (+ SE, n = 6) vertical daytime position of *Chaoborus* larvae in a stratified water column. The negative control treatment contained aged tap water, the control water extract contained aged tap water spiked with control water extract and the fish treatment consisted of aged tap water spiked with an extract of fish incubation water (*Rutilus rutilus*). * indicates a significant difference between treatment and negative control.

The subsequent experiment was conducted to test the ability of *Chaoborus* larvae to perceive their prey *Daphnia* chemically. *Daphnia* incubation water, applied either exclusively in the lower or upper stratum of the experimental tube, had a significant effect on the mean daytime residence depth of the *Chaoborus* larvae (Fig. 3, t-test: $t(20) = -5.23$, $p < 0.00005$). In tubes with *Daphnia* incubation water in the lower stratum of the tubes, *Chaoborus* larvae were attracted to a deeper daytime residence depth (65 cm) with lower mean temperature (13.7 °C) compared to tubes with *Daphnia* incubation water on top (35 cm and 18.4 °C). This

experiment does not include a negative control without any *Daphnia* incubation water added to either top or bottom. However, we included independent negative controls in three other experiments of this study (see Figs. 2, 5 and 6), which did not differ from one another (one-way ANOVA $F_{2, 23} = 0.631$ $p = 0.5412$), and all ranged between 38 cm and 43 cm. Thus it is reasonable to assume, that a vertical position around 40 cm is the common residence depth for *Chaoborus* larvae in negative controls this experimental setup. This also applies to the experiment depicted in Fig. 4.

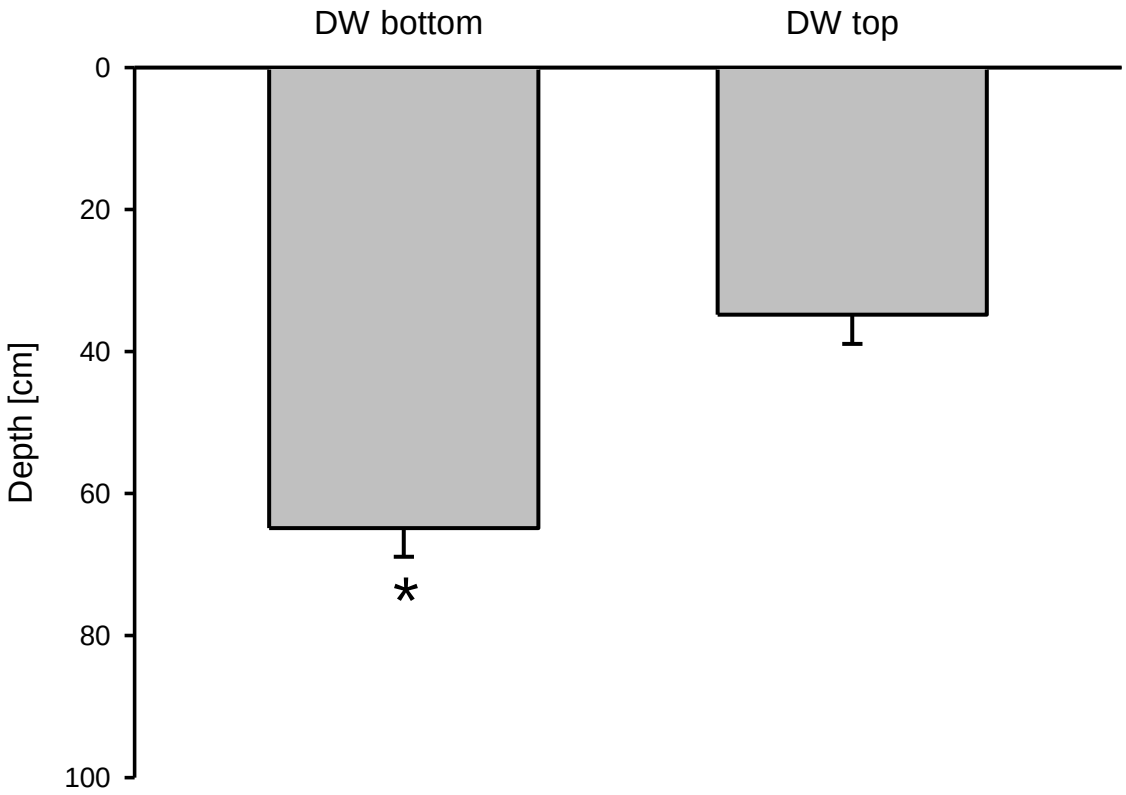

Figure 9: Mean (+ SE, n = 11) vertical daytime position of *Chaoborus* larvae in a stratified water column. The experimental tubes contained *Daphnia* incubation water either in the lower (DW bottom) or the upper (DW top) part. The remaining part of the water column contained control water. * indicates a significant difference between DW bottom and DW top.

In order to test how the simultaneous presence of chemical cues from fish and from *Daphnia* would affect the vertical position of *Chaoborus* larvae, we exposed *Chaoborus* larvae to *Daphnia* incubation water in the upper or lower stratum of the water column, and in half of the treatments this *Daphnia* incubation water was additionally spiked with extract of fish incubation water (Fig. 4). A one-way ANOVA revealed significant differences in residence depth of *Chaoborus* larvae ($F_{3, 36} = 13.857$; $p < 0.00005$). As in the earlier experiment (Fig. 3) DW top and bottom resulted in *Chaoborus* larvae residing at significantly different depths (DW top at 40 cm compared to DW bottom at 70; $p < 0.0005$ after Tukey). Additional spiking with the extract of fish incubation water resulted in a significant shift of the position of the larvae from 40 cm in DW top to 70 cm in 'DW top + fish' ($p < 0.0005$ after Tukey). In the DW bottom treatment the spiking with extract of fish incubation water had no effect on the depth or residence (70 cm in DW bottom, 65 cm in DW bottom + fish, $p = 0.879$, after Tukey). The mean positions of *Chaoborus* larvae in the DW bottom treatment spiked with fish were significantly deeper than those in the DW top treatment (Tukey's HSD: $p < 0.001$).

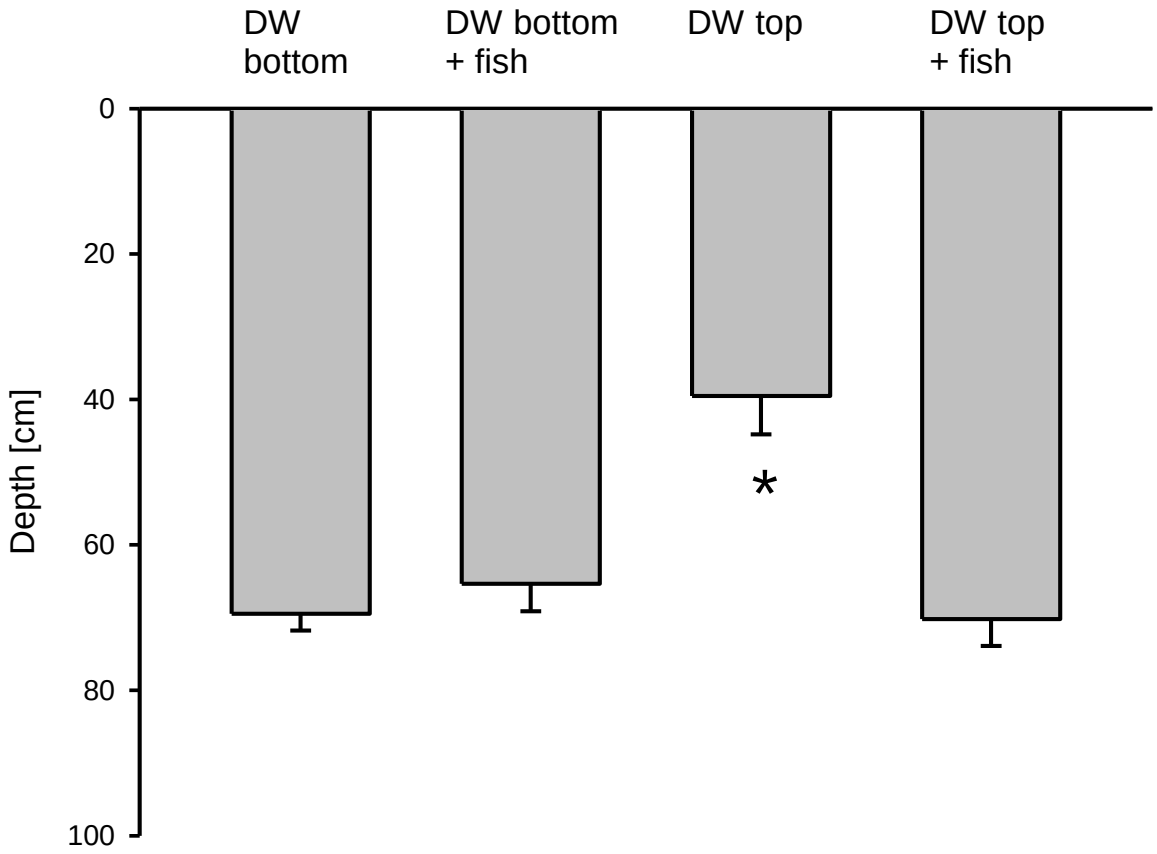

Figure 10: Mean (+ SE, n = 11) vertical daytime position of *Chaoborus* larvae in a stratified water column. The tubes contained *Daphnia* incubation water either in the lower (DW bottom) or the upper (DW top) part both with and without additionally applied fish incubation water extract. The remaining part contained only control water. * indicates a significant difference from the other treatments.

In order to test if it would be possible to obtain a concentrated and stable biologically active extract of the *Daphnia* incubation water, this water was subjected to extraction by a lipophilic solid phase (C_{18}-SPE). Therefore, both control water and *Daphnia* incubation water were passed through a C_{18} cartridge, and the permeates were subjected to a bioassay with control water and *Daphnia* incubation water as negative and positive control, respectively. *Daphnia* incubation water applied to the lower stratum of the water column induced a significantly deeper daytime residence (66 cm) of *Chaoborus* larvae in the stratified water column than control water (38 cm), control permeate (38 cm) and *Daphnia* incubation water permeate (38 cm) (Fig. 5, one-way ANOVA: $p < 0.0005$; $F_{3, 26} = 27.00$ followed by Tukey's HSD). The

vertical position of the larvae differed neither between the two permeates (Tukey's HSD: p = 0.999) nor between either permeate and the negative control (Tukey's HSD: p = 0.998 for control permeate and 0.999 for *Daphnia* water permeate). This suggested that the biological activity of the *Daphnia* incubation water had been retained by the lipophilic surface.

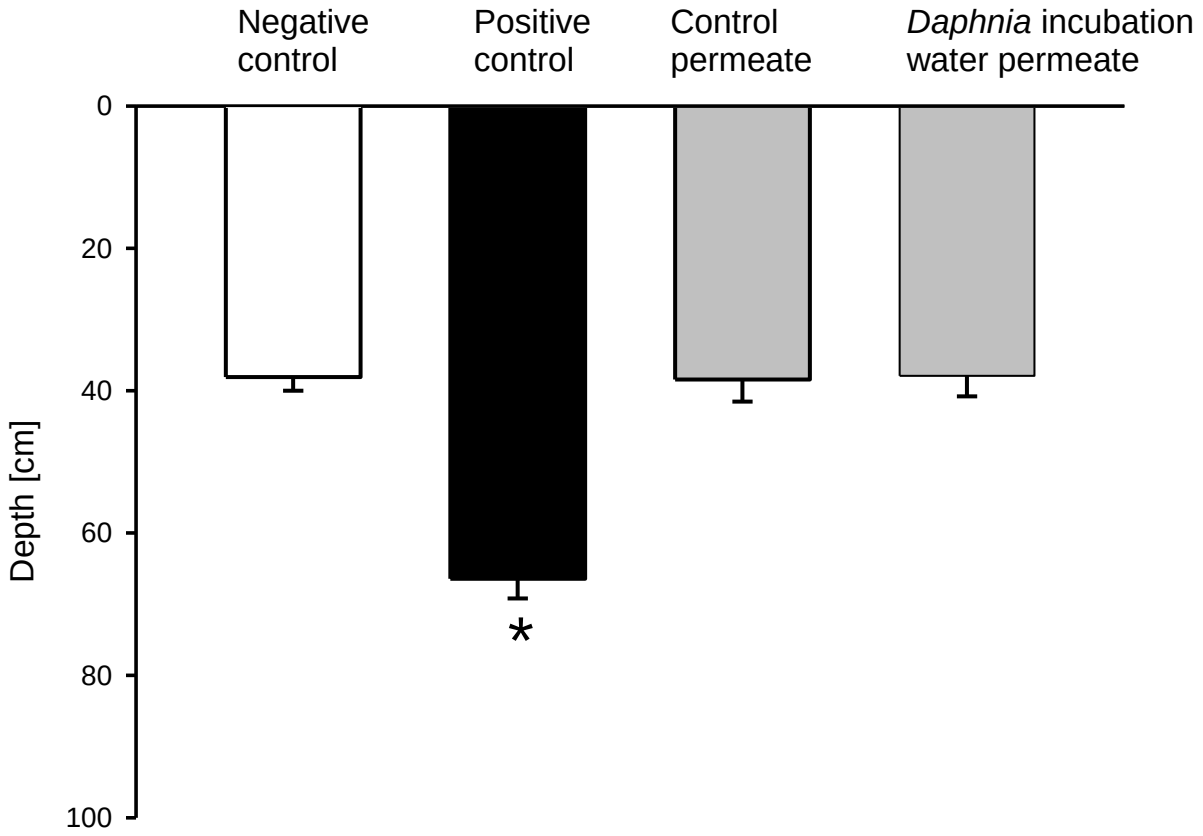

Figure 11: Mean (+ SE, n = 10) vertical daytime position of *Chaoborus* larvae in a stratified water column. The tubes contained control water as negative control, *Daphnia* incubation water as positive control, and control water or *Daphnia* incubation that had passed through a lipophilic solid-phase cartridge (control permeate and *Daphnia* incubation permeate). * indicates a significant difference from the other treatments.

In order to test for a putative retention of this activity, the respective SPE-cartridge was eluted with methanol, which resulted in the respective extract. A subsequent bioassay revealed significantly different mean daytime residence of *Chaoborus* larvae (Fig. 6, one-way ANOVA: $p < 0.0005$; $F_{3, 35} = 27.00$ followed by Tukey's HSD). The mean daytime residence

of *Chaoborus* larvae in a stratified water column in the negative control (38 cm) differed significantly from the positive control (69 cm, Tukey's HSD: $p < 0.0005$) and the *Daphnia* incubation water extract (62 cm, Tukey's HSD: $p < 0.0005$) but not from the control extract (40 cm, Tukey's HSD: $p = 0.969$). The response to *Daphnia* incubation water extract did not differ from that to the positive control (Tukey's HSD: $p = 0.467$) but was different from the control extract (Tukey's HSD: $p < 0.005$).

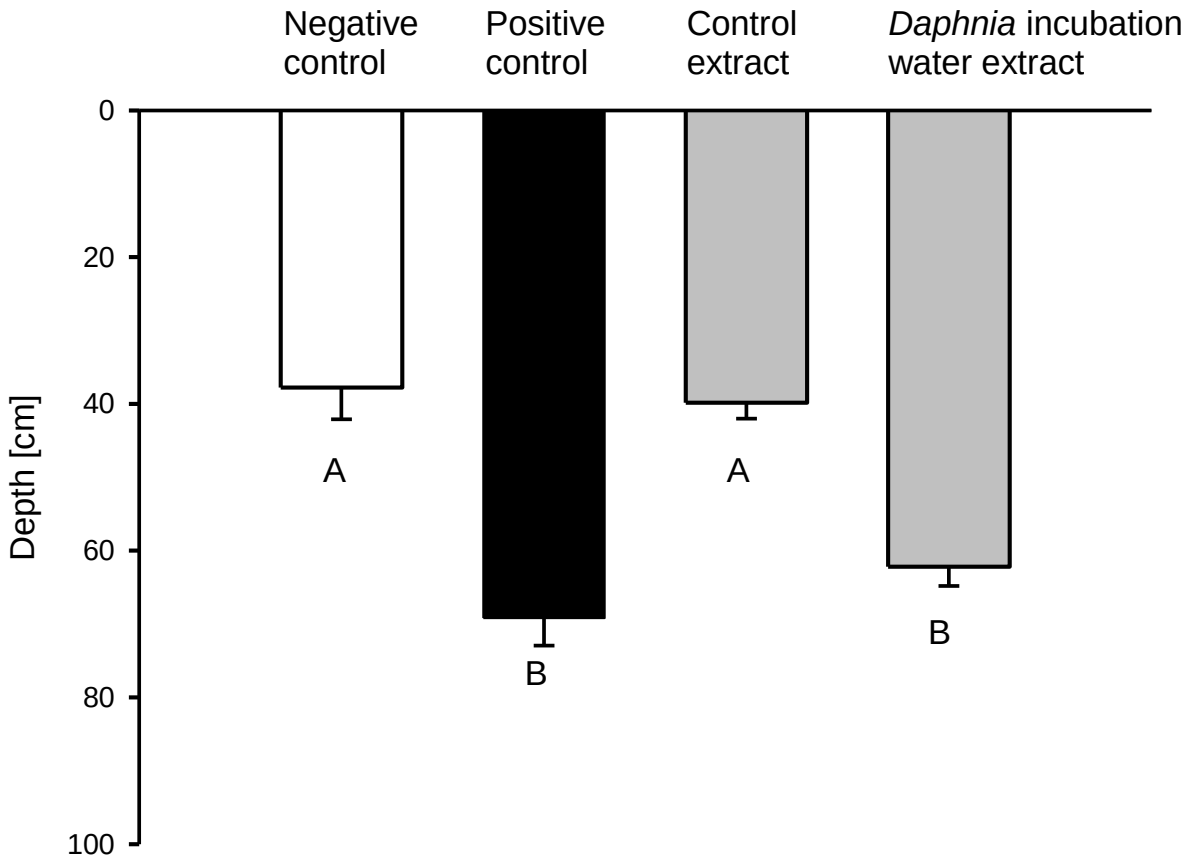

Figure 12: Mean (+ SE, n = 10) vertical daytime position of *Chaoborus* larvae in a stratified water column. The tubes contained control water as negative control, *Daphnia* incubation water as positive control, and the C_{18} extracts of both extracted control water and *Daphnia* incubation water. Different characters indicate significantly different groups.

Discussion

The results of this work show for the first time a chemically mediated prey induced vertical migration in *Chaoborus* larvae. The kairomone can be enriched from *Daphnia* incubation water by C_{18}-based solid phase extraction. Further, results from previous studies concerning the induction of DVM in *Chaoborus* could be reproduced by applying fish kairomone extract. The simultaneous exposure of *Chaoborus* larvae to kairomones from prey (*Daphnia*) and predator (fish) highlighted the complexity of multi-trophic environments with *Chaoborus* larvae avoiding *Daphnia* incubation water that was spiked with fish incubation water extract and applied exclusively in the top water layer and ignoring the kairomone of their predator if it was applied to the bottom layer.

When *Chaoborus* larvae follow the chemical signal(s) released by *Daphnia* prey in the hypolimnion, the benefit of an increased encounter probability with prey is traded-off by lower developmental rates (Buns and Ratte, 1991) and by slower attack rates and longer handling times caused by lower temperatures in the deeper strata (Spitze, 1985). The fact, that *Chaoborus* does not only induce vertical migration in a variety of different zooplankton species (Lagergren et al., 2008) but also uses chemical cues of prey as foraging kairomones shows once more how complex and sophisticated their predator-prey interactions are. Such a chemically induced change in behaviour of *Chaoborus* due to dissolved cues of zooplankton prey has yet only been reported with respect to an increase of movement frequencies (Berendonk and Obrien, 1996).

It is reasonable to assume that the attraction of *Chaoborus* larvae to the water layer with *Daphnia*-borne chemical cues represents an adaptive response. Larvae of *Chaoborus* are a sit-and-wait predator and are known to mainly rely on tactile stimuli for the short-distance attack of prey. Therefore, unlike planktivorous fish, the predation efficiency of *Chaoborus* larvae on zooplankton is not affected by the ambient light level (Swift and Forward, 1981). Assuming that attraction of *Chaoborus* larvae to the layer of *Daphnia*'s residence will increase the

probability of encountering *Daphnia*, the chemical cues released by this prey may be termed foraging kairomone (Ruther et al., 2002).

In this work, water with a density of 300 to 450 *D. magna* L^{-1} individuals was used for the preparation of prey conditioned water. This density clearly exceeds mean densities of *D. magna* in lakes and ponds, however, the distribution of *Daphnia* is known to be patchy, and the occurrence of *Daphnia* swarms in nature is well known. This has been attributed to the patchy distribution of food alga and horizontal migration in response to *Chaoborus* larvae (Kvam and Kleiven, 1995), which led to the formation of dense swarms of *Daphnia longispina* with up to 4000 individuals per liter in the litoral zone, which is even an order of magnitude denser than the abundances used here for the preparation of *Daphnia* incubation water.

Generally, the strike efficiency of *Chaoborus* larvae increases with increasing prey densities up to a density of 50 prey per litre (Jeschke and Tollrian, 2005). However, the formation of dense prey swarms can lead to confusion in the predator, resulting in a reduced strike efficiency in *Chaoborus obscuripes* larvae when facing *Daphnia obtusa* abundances of 50 individuals per litre or higher (Jeschke and Tollrian, 2005). On the other hand, this may explain why in treatments with *Daphnia* incubation water applied to the lower quarter of the experimental tubes, *Chaoborus* larvae stayed at greater depth than in controls but did not enter the layer with the incubation water. Instead the larvae lingered in the transition zone of incubation water and control water, where the "smell" of prey is probably only present in traces and so the virtual abundance of *Daphnia* does not suggest a too dense swarm. This is in line with the conclusions of Jeschke and Tollrian (Jeschke and Tollrian, 2007), who proposed, that confusion of a predator appears to decrease if it is easier for a predator to single out individual prey. This ability to single out individuals includes the ability to concentrate on individuals at the edge of a swarm and to reduce the field of attack.

It has been shown earlier that chemical signals from fish induced vertical migration in *Chaoborus* (Dawidowicz et al., 1990). Here we show that these compounds can be enriched from fish incubation water by lipophilic solid phase extraction using a protocol that is identical to the enrichment of the fish-borne kairomone that induces DVM in *Daphnia* (von Elert and Loose, 1996). The chemical structure of the kairomone inducing DVM in *Daphnia* has still not been fully elucidated (von Elert, 2012), and it remains to be seen if *Chaoborus* responds to the same kairomone as *Daphnia*.

Furthermore, we found that predator kairomones do not prevent *Chaoborus* larvae from descending to deeper and thus darker water layers, if these layers are chemically signalling the presence of *Daphnia* prey. This can be explained by the fact that the risk of predation by an optically hunting predator such as perch is conceivably low in near darkness. On the other hand, *Chaoborus* as a tactile non-optically hunting sit-and-wait-predator does not suffer any disadvantages from foraging in darkness (Swift and Forward, 1981). However, fish kairomone still exhibits its repelling effect, if applied exclusively in the top layer of the water column together with *Daphnia* incubation water. The danger of predation by fish overrides the attraction by the smell of prey, if predation risk in illuminated water layers is high. As a consequence *Chaoborus* larvae minimize spatial overlap with the layer of predator residence by migrating to deeper water layers without fish kairomone to avoid predation. This pattern is in accordance with the well established pattern of diel vertical migration of *Chaoborus* larvae in the field (e.g. Luecke, 1986), which is believed to be induced by fish.

In particular the experiments by Dawidowicz et al. (Dawidowicz et al., 1990) have led to the general opinion that the well known pattern of DVM in *Chaoborus* larvae in the field is induced by kairomones released by fish. However, the results of this study strongly suggest that chemical cues released from *Daphnia* that are seeking refuge from fish predation in the hypolimnetic layer of a stratified lake might contribute to this pattern of DVM in *Chaoborus*.

The experimental setup used in this study involved actively feeding *Daphnia* for the preparation of *Daphnia* incubation water. Generally, in natural lakes, the hypolimnion is considered to contain less edible phytoplankton for *Daphnia* than the epilimnion. However, this is not always the case, since lakes may have a deep chlorophyll maximum (DCM) and the seston of the DCM does not provide growing conditions that are worse than that of surface waters (Winder et al., 2003). However, contrary to widely accepted theory, Williamson et al. (Williamson et al., 1996) made observations which proved strata below the epilimnion to contain food of even better quality than the surface waters, attributing DVM solely to temperatures and predation risk.

The findings of this work have demonstrated that the biological activity of *Daphnia* incubation water can entirely be removed from active water and can be fully recovered by subsequent elution of the C_{18}-SPE-cartridge. This is in line with other known aquatic kairomones, which have been shown to be extractable by C_{18} SPE. For example von Elert and Loose (1996) produced enriched extracts of fish kairomone, as did Tollrian and von Elert (1994) with *Chaoborus* kairomone. The biological activity has proven to be stable so that lipophilic SPE can be applied to concentrate the activity by several orders of magnitude, which allows for the production of a highly concentrated extract that can be used as a standardized biological activity in subsequent experiments. Also, these results allow for further chemical characterization of the infochemical.

In addition, this work contributes to the understanding of multitrophic predator-prey systems in general and illustrates the sophistication with which *Chaoborus* larvae chemically monitor their surroundings. They utilize kairomones to differentiate between potential prey and predators. The results indicate, that *Chaoborus* larvae can weigh the consequences of following the smell of prey into differently dangerous water bodies, so that the observed vertical position in the water column seems to result from balancing benefits and specific trade-offs.

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Concluding remarks and perspective

Over the course of the last decades, *Daphnia* has become one of the most important model organisms for freshwater ecology (Lampert 2006). This is especially true for interaction-based research, as *Daphnia* is both predator and prey at the same time.

As a predator, or more precisely grazer in the case of *Daphnia*, they feed on phytoplankton on a broad size range limited by their filter apparatus. As a prey species, *Daphnia* is food source for vertebrate and invertebrate predators. Among invertebrates, the gape-limited sit-and-wait-predator *Chaoborus* is known to prey heavily on *Daphnia*. The presence of *Chaoborus*, which is mediated via predator-borne chemical stimuli, known as kairomones, induces different types of defences (i.e. morphological or behavioural defences as well as changes in life-history) in *Daphnia* which have been shown to decrease mortality due to predation.

As a morphological defence, *Daphnia pulex* develops neck-teeth at the back of the head, which were shown to increase survival by up to 45 % (Hammill *et al.* 2008). Neck-teeth do not influence the evasion efficiency of *Daphnia* (*Chaoborus* larvae do not have a higher percentage of missed attacks on *Daphnia* with neck-teeth than on *Daphnia* without), but have been shown to increase the escape efficiencies of already captured *Daphnia* (Havel and Dodson 1984). As *Chaoborus* typically handles *Daphnia* by the head end, spike-like structures at this part of the body probably interfere mechanically with the prey capturing apparatus of *Chaoborus* (cited according to (Havel and Dodson 1984)).

Many aspects of neck-teeth such as efficiency of defence (Havel and Dodson 1984), costs (e.g. (Riessen 2012)) or the negative influence of changing natural conditions, such as reduced calcium levels due to atmospheric acid deposition, on the strength of induced neck-teeth (Riessen *et al.* 2012) have been thoroughly elucidated. Others focused on the neuronal signalling mediating the anti-predator response of *Daphnia* (Weiss *et al.* 2012) or the identification of the precise kairomone-sensitive period during embryonic development (Naraki and others 2013). However, only very few studies have been conducted on the afore

mentioned aspect of *Daphnia* being a keystone species and both predator and prey at the same time. Black and Dodson (1989) performed life-history experiments under limiting and saturated food conditions and in the absence and presence of *Chaoborus* kairomone with *D. pulex* in order to identify the demographic costs associated with neck-teeth induction. A similar attempt was conducted by Pauwels *et al.* (2010) with *D. magna* and fish kairomones. Yet, to my knowledge, these are the only two studies investigating the influence of food on the inducible defences of *Daphnia*. And even those two studies ignored the fact that there is never only one edible phytoplankton species in natural freshwater ecosystems, as is typical standard procedure for lab cultures, which is only varied concerning the available quantity. Ponds and lakes are filled with a vast variety of algae of an as diverse spectrum of food quality, which *Daphnia* is not able to selectively feed on due to their physiological status as an unselective filter feeder. **Chapter 1** addresses the question if and how maternal food of a given quality can influence the strength of neck-teeth induction. In general, there were two conceivable scenarios if food quality did indeed have an impact on inducible morphological defences. Low quality food could either lead to an increase or a decrease of exhibited strength of the defence. A hypothetical decrease could have been explained by metabolic costs imposed by the induction of the defence and the trade-off to invest resources rather into somatic or population growth than into defence. However, as shown in **Chapter 1**, the cultivation of *Daphnia* mothers on low quality food led to an increase in the strength of neck-teeth induction in juveniles compared to juveniles of mothers reared on food of medium or high quality. Further, growth experiments demonstrated that juveniles of *Daphnia pulex* needed a longer time to reach the third larval instar if their mothers were reared on low quality food. As *Chaoborus* is a gape-limited predator, juvenile *Daphnia* have a decreasing predation risk with increasing size in juvenile instars. Thus, a longer time needed to reach the third juvenile instar results in a longer time window of high predation risk. Therefore, an increased morphological defence at low food is assumed to compensate for the longer time of suffering

an increased predation risk due to the smaller body size. Beyond the results of **Chapter 1**, the aspect of a quantified effective defensive benefit of a given neck-teeth induction deserves more investigation. Predation trials on *Daphnia* morphs with differently induced neck-teeth defences could provide deeper insights into the complex coupling of food quality and morphological defence.

Beyond this, I identified an omega-3 poly-unsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA), to be responsible for the suppression of neck-teeth induction in food of high quality by supplementing low quality food with liposomes containing different PUFAs. Other tested fatty acids (arachidonic acid, vaccenic acid) did not affect neck-teeth induction. However, EPA has previously been shown to increase juvenile growth rates of *Daphnia* grown on EPA deficient food (von Elert 2002), thus an EPA induced increase in juvenile growth rate makes the necessity for an increased metabolic investment into a morphological defence obsolete.

The results of **Chapter 1** show for the first time how maternal dietary quality affects the strength of a morphological anti-predator defence in a prey species and thus point out how important the identity of the fed food algae is for *Daphnia* lab cultures, especially, if results of different studies with deviating culturing methods on a specific topic are to be comparatively discussed.

Chapter 2 changes the perspective on the topic of neck-teeth induction by dealing with the question of how this morphological defence is regulated on the gene level. Although ultimately supposedly regulated via endocrinal pathways, hormone titres in organisms of approximately one millimetre body size are hard to measure. Thus, an indirect approach via the measurement of relative gene expression levels of hormone pathway related genes via qPCR had to be considered. Past studies focused on the juvenoid signalling pathway (e.g. (Miyakawa *et al.* 2010) or (Dennis *et al.* 2014)) and found supposedly evidence for its involvement. However, experimental setups resulted in rather imprecise sampling time with

probably neck-teeth unrelated gene expression profiles (Miyakawa *et al.* 2010) or a flawed way of extracting kairomone for neck-teeth induction, probably resulting the enrichment of *Chaoborus*-borne juvenile hormone und thus leading to gene expression profiles which can no longer be unambiguously attributed to the kairomone itself (Dennis *et al*. 2014). Further, neck-teeth expression was quantified in neither study, leaving the relative gene expression of the candidate genes unconnected to the actual strength of the morphological defence. In **Chapter 2**, I applied an improved method with a very narrow time window and a kairomone enrichment method which was based on the extraction of *Chaoborus* incubation water rather than extracting whole larvae tissue in order to minimize the enrichment of juvenile hormones contained within the larval bodies. Further, I introduced a "clonal gradient". Instead of working with a single *D. pulex* clone only, three different *D. pulex* clones were chosen, due to their significantly deviating neck-teeth inductions if exposed to equal amounts of *Chaoborus* kairomone. Thus it was possible to correlate gene expression profiles of the three clones with their respective neck-teeth induction with and without the exposure to *Chaoborus* kairomone.

Surprisingly, the results presented in **Chapter 2** indicate no involvement of the juvenile hormone pathway or the insulin signalling pathway whatsoever. Although a possible involvement of these hormonal pathways and the proposed downstream genes cannot be completely excluded, since only a small set of genes was investigated concerning a kairomone induced upregulation, the results of past studies have to be carefully re-evaluated, in order to avoid future projects being on the wrong track from the beginning on. In addition, it becomes clear that the elucidation of the regulatory mechanisms underlying the induction of neck-teeth is far from being concluded.

Furthermore, additional insights into the identity of other neck-teeth related genes could be obtained. Otte *et al*. (2014) used a proteome based approach to detect an increase of abundance of chitin deacetylases due to an exposure of *Daphnia magna* to kairomone derived from *Triops cancriformis*. This kairomone is known to induce an increased bulkiness in

Daphnia functioning as a morphological defence against predation. **Chapter 2** contains data clearly correlating gene expression levels of two chitin deacetylases in *D. pulex* with the respective neck-teeth expression quantified for the experimental animals directly prior to RNA extraction and thus connecting the regulation of genes directly to the exhibited strength of neck-teeth induction. This does not only indicate the shared genetic background of morphological defences of different *Daphnia* species but also provides a basis for future research in terms of an alternative starting point when unravelling potential regulatory networks.

When investigating predator-prey interactions in general and in *Daphnia* in particular, most studies seem to focus on aspects concerning the prey species. However, understanding only one side of the coin is not sufficient for the comprehension of the entire complexity which makes up the whole picture of a predator-prey relation. With *Daphnia* being a model organism, not only the predator induced defences in *Daphnia* are important, but also the means and strategies of the predator to antagonizing the defences have to be uncovered, in order to be able to obtain ecologically applicable results. In **Chapter 3** I investigated if and how the presence or absence of *Daphnia* cues influences the vertical position of *Chaoborus* larvae in the water column by conduction behavioural assays in a temperature and light controlled plankton organ. Beyond this, and taking *Chaoborus* position as both predator and prey in freshwater food webs into account, the influence of fish kairomone indicating the presence of a top predator was explored, when temporally and spatially overlapping with *Daphnia* incubation water. Earlier studies already demonstrated an induced vertical migration of *Chaoborus* larvae due to the exposure to fish kairomone (Dawidowicz *et al*. 1990). However, it was still unclear, whether *Chaoborus* can not only perceive chemical substances released a predator or also by potential prey. In addition, I investigated the effects of the combined presence of both kairomones released by *Daphnia* and fish, and if this effects *Chaoborus* differently under different light regimes.

The results clearly prove the existence of a foraging kairomone released by *Daphnia*. When incubation water, wherein *Daphnia* were kept and fed for 24 hours was applied either at the top or bottom of a stratified water column, *Chaoborus* larvae positioned themselves higher or lower, following the "smell" of their prey, indicated by chemical stimuli contained in the incubation water. Beyond this, the results of **Chapter 3** demonstrate that if predator kairomone is simultaneously applied with *Daphnia*-borne foraging kairomone either at the top or bottom layer of the stratified water column, the behavioural response of *Chaoborus* was shown to be light dependent. If foraging and predator kairomone co-occurred in the top strata of the water column, where visibility was higher, *Chaoborus* larvae exhibited the typically anti-predator response known from previous studies and ignored the presence of the foraging kairomone. To be specific, they took up position at a lower depth compared to kairomone-free control treatments, in order to minimize spatial overlap with optically hunting predators during daytime light conditions. On the other hand, if the presence of the optically hunting predator co-occurred with the foraging kairomone in the lower and thus darker strata of the plankton organ, the vertical position of *Chaoborus* larvae did not differ from predator kairomone free treatments, suggesting that *Chaoborus* is able to assess the potential risk imposed by an optically hunting predator as non-significant in the dark and thus not altering its behavior. These results prove for the first time the existence of a foraging kairomone released by *Daphnia* and illustrate the complexity of predator-prey interactions in multitrophic foodwebs by demonstrating how *Chaoborus* can differentiate between the mere presence or absence of two different kairomones and adjust its behavioural response to these two kairomones with regard to ambient light conditions.

Finally, **Chapter 3** contains data elucidating the chemical properties of the *Daphnia*-borne foraging kairomone. The chemical cue was shown to be extractable via C_{18} solid phase extraction, suggesting a lipophilc nature of the molecule(s).

This present dissertation applied both classical ecological approaches such as life history experiments, morphological investigations and behavioural assays as well as qPCR analyses to add a new depth to the knowledge of reciprocal mechanisms regulating the interactions of the model keystone species *Daphnia* with both its food and predator. The results of this study do not only demonstrate how defining the quality of maternal food is for the prospective anti-predator defence of the offspring but immediately present a substance determining this predator associated aspect of food quality. Thus, an important linkage between the two fields of food quality research and anti-predator defence could be accomplished that hopefully helps to further elucidate the role of *Daphnia* as a model organism in freshwater ecosystems. The qPCR data facilitates the reconsideration of previously suggested endocrine pathways thought to be responsible for the regulation of neck-teeth and at the same time offers new candidate genes associated with neck-teeth induction. Finally, the uncovering of the foraging kairomone released by *Daphnia* clarified that predator-prey relations are not one-way streets but are rather based upon reciprocal interactions in the context of an evolutionary arms race whereby each species tries to be one step ahead of the other.

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Abstract

The crustacean grazer and model organism *Daphnia* is prey to both vertebrate and invertebrate predators and thus in a keystone position for the energy transfer in standing freshwater ecosystems. It exhibits a high degree of phenotypic plasticity in a vast variety of traits, including inducible defences against predators such as changes in life-history, morphology or behavior. These defences are likely to impose metabolic costs, as they are not continually expressed but only in the presence of a reliable chemical stimulus, a so called kairomone, released by the respective predator indicating its presence and thus the imminent danger of predation.

This present study is focussed on the elucidation of different mechanisms connected with or underlying the reciprocal predator-prey interactions of *Daphnia* and the aquatic larvae of the phantom midge *Chaoborus*, an important gape-limited invertebrate predator, both under the impact of one lower and one higher trophic level. As *Daphnia* is known to feed unselectively on phytoplankton of a given size range, they are highly depended on the overall food quality of the available phytoplankton. I investigated the influence of three different food algae on the capability of *Daphnia pulex* to induce a morphological defence called neck-teeth and related the strength of the induction to the juvenile growth rate of the first two juvenile instars with was conditional on the quality of the food. The quality of the respective algae as a food for *Daphnia* was determined by observing the time juveniles took to complete two moulds. As low quality food leads to slower growth, a higher neck-teeth induction was observed, which was presumably to compensate for a longer time needed to outgrow the optimal prey size range of *Chaoborus*. A poly-unsaturated fatty acid (eicosapentaenoic acid - EPA) was identified as molecule responsible for the suppression of neck-teeth induction in high quality food, which has previously been shown to lead to an increased juvenile growth rate when provided to *Daphnia* reared on low quality food.

Additionally, I investigated relative gene expression of candidate genes putatively involved in the regulation of neck-teeth induction. No evidence for an involvement of the presumably involved juvenile hormone / insulin pathway could be found, as *Chaoborus* kairomone did not induce any differential gene expression of candidate genes as reported by previously published studies. I applied a stricter method for both RNA sampling and kairomone enrichment in order to avoid potential flaws of preceding studies on this topic and to produce more substantial and reliable results. Further, the relative expression of two new candidate genes, i.e. chitin deacetylases, were found to be significantly correlated with the neck-teeth induction in three different *D. pulex* clones, all exhibiting different neck-teeth inductions with corresponding gene expression levels.

Finally this study presents for the first time data on a foraging kairomone released by *Daphnia*, which was shown to induce a change in the vertical position of *Chaoborus* larvae in the water column towards the strata containing the kairomone. Additionally, the simultaneous presence of fish kairomone induced different behavioural responses from *Chaoborus*. If applied in the top strata of the water column where visibility was high, the effect of the predator kairomone overruled the effect of the foraging kairomone. If applied to the lower strata of the water column where visibility was lower, the effect of foraging kairomone overruled the effect of the predator kairomone. These results clarify, how *Chaoborus* can assess potential costs and benefits resulting from the presence of different kairomones and the respective surrounding light conditions.

Zusammenfassung

Der Modelorganismus *Daphnia* ist Beute für vertebrate und invertebrate Räuber und befindet sich somit in einer Schlüsselposition für den Energietransfer in stehenden limnischen Ökosystemen. Daphnien zeigen ein hohes Maß an phänotypischer Plastizität in einer großen Zahl an Merkmalen, darunter auch induzierbare Verteidigungen gegen Fraßfeinde wie zum Beispiel Änderungen der Life-History, der Morphologie oder des Verhaltens. Diese Verteidigungen sind höchstwahrscheinlich mit metabolische Kosten verknüpft, da sie nicht konstitutiv exprimiert werden sondern nur in Gegenwart eines verlässlichen chemischen Signales, eines sogenannten Kairomones, das von dem entsprechenden Fraßfeind ausgesandt wird und dessen Anwesenheit und somit eine drohende Gefahr der Prädation anzeigt.

Die vorliegende Studie ist auf die Aufklärung verschiedener Mechanismen fokussiert, die mit den wechselseitigen Räuber-Beute Beziehungen von Daphnien und der aquatischen Larve der Büschelmücke *Chaoborus* in Verbindung stehen, jeweils unter Miteinbeziehung einer höheren und niedrigeren trophischen Ebene. *Chaoborus* ist ein Maulspalten-limitierten invertebraten Fraßfeind von Daphnien. Da sich Daphnien unselektiv von Phytoplankton eines bestimmten Größenspektrums ernähren, sind sie abhängig von der Komposition und Qualität des umgebenden und somit verfügbaren Phytoplanktons. Ich untersuchte den Einfluss von drei verschiedenen Futteralgen auf die Fähigkeit von *Daphnia pulex* eine morphologische Verteidigung, sogenannte Nackenzähne, auszubilden. Dafür setzte ich die Stärke der Ausprägung der Nackenzähne mit der juvenilen Wachstumsrate in den ersten beiden juvenilen Stadien in Bezug, welche von der Qualität des jeweiligen Futters abhing. Die Futterqualität wurde daran bemessen, wie lange die juvenilen Daphnien bis zur vollendung der zweiten Häutung benötigten. Da Futter niedriger Qualität zu langsamerem Wachstum führte, wurde eine stärkere Nackenzahn Induktion beobachtet, die wahrscheinlich die verlängerte Zeit kompensieren sollte, in der sich die Daphnien in der optimalen Beutegröße von *Chaoborus* befanden. Eine mehrfach ungesättigte Fettsäure (Eicosapentaensäure - EPA)

wurde als, für die Erniedrigung der Nackenzahn Induktion in Futter von hoher Qualität verantwortliche Substanz in dem Futter von hoher Qualität identifiziert. Zuvor konnte bereits gezeigt werden, dass EPA in Daphnien, die auf Futter von minderer Qualität gehältert wurden, zu einer Erhöhung der somatischen Wachstumsraten führt.

Zusätzlich untersuchte ich die relative Genexpression von Kandidaten Genen die vermutlich verantwortlich für die Regulation von Nackenzähnen sind. Es konnten keine Beweise für eine Beteiligung von vormals verantwortlich gemachten Hormon-Signalwegen (Juvenoid Hormon und Insulin Signalweg) gefunden werden, da die Anwesenheit von *Chaoborus* Kairomon keine differentielle Genexpression der Kandidatengene bewirkte, wie von vorangegangenen Studien gezeigt worden war. In der vorliegenden Arbeit wurden strengere und verfeinerte Methoden sowohl für die RNA Probenahme als auch für die Kairomon Anreicherung verwendet, um potentielle Fehlerquellen der vorangegangenen Studien zu diesem Thema zu vermeiden und substantiellere und verlässlichere Daten zu erheben. Weiterhin konnte gezeigt werden, dass die Genexpression von zwei Chitindeacetylasen signifikant mit der quantifizierten Nackenzahninduktion von drei verschiedenen *D. pulex* Klonen korreliert war, die alle eine unterschiedlich starke Ausprägung der induzierten Verteidigung zeigten.

Ferner zeigte diese Studie zum ersten Mal, dass Daphnien ein Furagierkairomon freisetzen, welches dahingehend eine Änderung der von *Chaoborus* Larven in der Wassersäule eingenommenen vertikalen Position induziert, dass sich diese näher an den kairomonhaltigen Schichten positionierten. Weiterhin löste die simultane Anwesenheit von Fisch Kairomon, differenzierte Verhaltensantworten bei *Chaoborus* aus. Wenn die beiden Kairomone in den oberen Wasserschichten präsent waren, in denen die allgemeine Sichtbarkeit hoch war, hob der Effekt des Prädatorkairomones die Wirkung des Furagierkairomones auf. Wurden beide Kairomone in den tieferen Schichten eingesetzt, wo die Sichtbedingungen generell schlecht sind, hob die Wirkung des Furagierkairomones die Wirkung des Prädatorkairomones auf.

Diese Ergebnisse verdeutlichen, wie *Chaoborus* potentielle Kosten und Nutzen von der Anwesenheit verschiedener Kairomone und den umgebenen Lichtverhältnissen ableiten kann.

Record of achievement

Chapter 1: Dietary quality affects the strength of a morphological anti-predator defence in *Daphnia*.

All results were performed by me or under my direct supervision. Christoph Effertz contributed by assisting during the 90 hours period of hourly sampling. Eric von Elert was involved in all technical discussions and discussions regarding desicisions and has critically read the manuscript. Patrick Fink provided useful conceptional ideas.

Chapter 2: Differential expression of candidate genes for neck-teeth induction in three *D. pulex* clones of varying responsiveness to *Chaoborus* kairomone

All results were exclusively performed by me or under my direct supervision. Eric von Elert was involved in all technical discussions and discussions regarding desicisions and has critically read the manuscript. Patrick Fink provided useful conceptional ideas.

Chapter 3: Prey induced vertical migration in *Chaoborus* larvae under different predator and light regimes.

All results were exclusively performed by me. Eric von Elert was involved in all technical discussions and discussions regarding desicisions and has critically read the manuscript.

Liste der Publikationen im peer-review Verfahren

[1] Christjani, M., Effertz, C., Fink, P., von Elert, E. Dietary quality affects the strength of a morphological anti-predator defence in *Daphnia*. *Current Biology* - under review.

[2] Christjani, M, Fink, P., von Elert, E. Phenotypic plasticity in three *Daphnia* genotypes in response to predator kairomone: no evidence for juvenile hormone signalling but for involvement of chitin deacetylases. *Mechanisms of Development* - under review.

[3] Christjani, M. & von Elert, E. (2014) Prey-induced vertical migration in Chaoborus larvae under different predator and light regimes. *Journal of Plankton Research* 37(1): 48-55 (doi:10.1093/plankt/fbu086)

[1] corresponds to chapter 1
[2] corresponds to chapter 2
[3] corresponds to chapter 3

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen und Abbildungen -, die anderen Werken in Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Eric von Elert betreut worden.

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